Cross-Talk Between Epigenetic Regulation And Mir-17–92 Cluster Expression In Idiopathic Pulmonary Fibrosis (IPF)

Dissertation

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By

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ABSTRACT

Interstitial lung disease/idiopathic pulmonary fibrosis (ILD/IPF) is the most progressive form of pulmonary fibrosis disorder and leads to death in patients afflicted. The etiology and pathogenesis of ILD/IPF in poorly understood with no known prevention or cure available.

ILD/IPF is associated with increased expression of certain fibrogenic genes such as CTGF, VEGF, GM-CSF, and TSP-1; however, the precise mechanism responsible for the increased gene and protein expression is not known. Since there are a large number of genes differentially expressed in the lungs of patients with ILD/IPF compared to controls, we speculated that elements like microRNAs (miRNAs) may be involved. miRNAs, also known as miRs, are small regulatory RNAs that alter gene expression by causing degradation, translational repression, or epigenetic regulation resulting in a change in protein expression. Changes in miRNA expression are associated with up to 30% of cancers (Volinia et. al., 2010) and a variety of other diseases (Nana-Sinkam et. al., 2009). Using miRNA profiling, we identified a specific miRNA cluster that was reduced in expression in ILD/IPF lung tissue compared to normal lung tissue and lung tissue from patients with chronic obstructive pulmonary disease (COPD), the miR-
17~92 cluster. The miR-17~92 polycistron cluster is ~ 1Kb located within the third intron of the open reading frame13q31.3 (C13orf25) which encodes six microRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1).

This locus is overexpressed in B-cell lymphomas and lung cancer. In ILD/IPF, miR-17~92 miRNA cluster targets genes, such as metalloproteinases, collagen, and transforming growth factor that are highly expressed.

Our preliminary data indicated decreased expression of the miR-17~92 miRNA cluster family members in the lungs from patients with ILD/IPF. Notably, miR-19b and miR-20a decreased proportionally to disease severity (and pulmonary function) of ILD/IPF. In ILD/IPF lung tissue, miR-19b and miR-20a expression was reduced and its predicted targets, CTGF, VEGF, TGF-β, TSP-1 and Ets-1 were up-regulated. Importantly, we noticed that the 5'-region of the promoter is heavily occupied with CpG Islands (>90%) and sought to define if this area was affected by methylation in ILD/IPF, as the mechanism causing down-regulation of the cluster expression in ILD/IPF. Our preliminary data suggested that this epigenetic silencing of miR-17~92 clusters was due to methylation of the promoter, and was increased with the severity of ILD/IPF. Indeed, we found this CpG island methylated in ILD/IPF and by relieving this methylation with 5'-aza-2'-deoxycytidine, enhanced cluster expression and decreased expression of the target genes. In addition, we found that the miR-17~92 cluster targeted DNA methyltransferases (DNMTs), which we believe are responsible for the methylation events in ILD/IPF. Interestingly, our data showed direct regulation of
miR-17, miR-19b, miR-20a and miR-92 on the 3'UTR of DNMT-1 in lung fibroblasts isolated from patients with IPF, indicating a negative feedback mechanism of the cluster to its own promoter though an epigenetic modifier.

In fact, inhibition of DNA methylation by 5'-aza-2'-deoxycytidine in our in vivo murine bleomycin-induced pulmonary fibrosis model recovered miR-17~92 cluster expression to that of normal lung tissue, rescued the animals’ lungs from fibrosis, and decreased fibrotic gene expression in their lungs. Thus, our data indicate that dysregulation of miRNA expression in IPF may contribute to the development and/or progression of the disease. 5’-aza-2’-deoxycytidine treatment, or miR-17~92 overexpression, could be a therapeutic intervention in ILD/IPF.
DEDICATION

This manuscript is dedicated to my family. To my mother who always prayed for me and sacrificed her life for me and others, to my father who encourages me through all those years and to my family who allowed me to go through this journey surrounded with their unconditional love and support.
ACKNOWLEDGMENT

I thank Ohio State University and the department of Molecular, Cellular and Developmental Biology for giving me the opportunity to attend their outstanding graduate program.

Exceptional thanks and my sincerest gratitude to my advisor, Dr. Clay Marsh, for his supervision, advice, guidance and support during my years in his lab. His passion for research, science, and having his standards high and being there for others has been the driving force and source of inspiration for everyone around him, especially me. He gave me the freedom to pursue a project that was different from what the lab had traditionally undertaken. He has provided me with persistent support and encouragement when it was most needed. He made sure that every one of his lab members pursuing a new idea or performing expensive experiments was never delayed or abandoned by providing us with the best resources and funds. I know I am so lucky to be his student and will always owe an enormous debt of gratitude to him for the rest of my life.

I would like to thank Dr. Melissa Piper for being patient, supportive and for challenging me to strengthen and build the skills to develop ideas, and design and complete experiments with the correct controls independently, as well as the talent to be decisive in problem solving and interpreting results.
I also thank my committee members, Dr. Virginia Sanders, Dr. Caroline Whitacre, Dr. Susheela Tridandapani, Dr. Jeanette Marketon and Dr. Terry Elton for their valuable time, support, contribution and recommendation.

My sincere gratitude goes to our lab members, especially Dr. Tim Eubank for always being there for me and others in both science and life as well.

I would like to thank Dr. Julie Roda for all her critical suggestions, support and her contribution in solving many problems I faced during my research.

I thank my classmate, Dr. Noura Ismail for her support. She always has been more than a lab mate to me. Also I would like to thank Yijie Wang and Valerie Wright for their great support and care.

I would like to record my appreciation to the animal core, especially Christie Newland and Arial Basye for helping me in our in vivo study.

I am blessed with my husband, and my kids, Ahmad, Adel, Heba and Haya. They have been a pillar of support in every step through my life especially through my research. Their love, patient, understanding and encouragement have been essential to keep me going and without them, I would have not been able to do it. I would never be able to express my great gratitude toward my parents Adel Dakhllalah and Intesar Al Masri for providing the best world they had to offer to ensure that I get the best environment and education in my life. I am thankful for the blessing, support, encouragement and love they offered me especially my beloved mom, who I wish she was here to see her effort has been
paid for and her wishes came true. She influenced my outlook toward life; she was always ready to help and she always will be by me in my journey.

I owe an enormous gratitude for my life time best friend, Eman Al-zaben, for her encouragement and being there for me. Last but not least, I thank my God for providing me all the above circumstances to make my dreams comes true.
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CHAPTER ONE: INTRODUCTION

I. IDIOPATHIC PULMONARY FIBROSIS

1-Characterization of Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is one of the most aggressive forms of interstitial lung disease and is characterized pathologically as temporally heterogeneous scarring and destruction of alveolar/capillary lung units. It is an inexorable progressive disease that most commonly occurs in 40 to 70 year olds with median survival less than three years after diagnosis (Nicholson et. al., 2000, Collard et. al., 2003; Kuwano et. al., 1996) and the incidence of the disease increases with age (Scott et. al., 1990; Mannino et. al., 1996). There is no known treatment for ILD/IPF and always results in death..

ILD/IPF has no distinctive geographic, race, gender or ethnicity distribution. However, it has been reported with unclear explanation that, the age-adjusted mortality rates are higher among whites than blacks, vary geographically, and differences in environmental and occupational exposure also appear to affect the mortality rate of ILD/IPF patients. In the United States, age-adjusted mortality rates due to pulmonary fibrosis are highest in the West and Southeast and lowest in the Midwest and Northeast. On the other hand, in the United Kingdom,
highest rates of mortality secondary to ILD/IPF occur in the industrialized central areas of England and Wales (Johnston et. al., 1990; Mannino et. al., 1996; ATS 2000, Fellrath and duBois 2003;).

ILD/IPF poses significant challenges clinically (Coultas et. al., 1994; Cherniack et. al., 1991). Pathologically, lung tissue obtained from patients with IPF is characterized histologically by features of common interstitial pneumonia (UIP) that include varying degrees of parenchymal inflammation (alveolitis), disruption of the pulmonary vascular bed, fibrosis of the pulmonary parenchyma, restrictive pulmonary dysfunction, abnormal gas exchange, chronic inflammation, accumulation of extracellular matrix components (ECM), resulting in the alveolar-capillary unit destruction and vascular remodeling (Ryu et. al., 1998; Bjoraker et. al., 1998).

2-Etiology and Pathogenesis

The etiology and pathogenesis of ILD/IPF leading to scarring of the lung tissue are poorly understood and remain unknown. The mortality rates from ILD/IPF are compared to other chronic diseases with same mortality (Table 1) (Kuwano et. al., 1996; Mason et. al., 1999).
Table 1. Mortality in ILD/IPF and Other Diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPF</td>
<td>50% at 5 yr</td>
</tr>
<tr>
<td>COPD FEV, 30% predicted</td>
<td>50% at 3 yr</td>
</tr>
<tr>
<td>COPD and long-term oxygen therapy</td>
<td>50% at 5 yr</td>
</tr>
<tr>
<td>Acute myocardial infarction (seen in emergency room)</td>
<td>25% at 5 yr</td>
</tr>
<tr>
<td>Coronary bypass surgery(3 vessels)</td>
<td>30% at 4 yr</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>85% at 5 yr</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>20% at 5 yr</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>15% at 5 yr</td>
</tr>
<tr>
<td>Cirrhosis with variceal bleeding</td>
<td>50% at 4 yr</td>
</tr>
</tbody>
</table>

ILD/IPF is characterized by abnormal pulmonary function studies with severe restriction (low Total Lung Capacity), impaired gas exchange and abnormal chest radiograph that worsen as ILD/IPF progresses. It is suggested that ILD/IPF is an epithelial-fibroblast disease (Willis et. al., 2005; Selman et. al., 2006), likened to a chronic non-healing wound resulting in matrix activation and failure of alveolar epithelial cells to repair the wound. As a result, fibroblasts are recruited to the area and induce fibro-proliferation, where fibroblast phenotype changes to become myofibroblasts producing collagen and other fibrogenic proteins (Selman et. al., 2001; Pardo et. al., 2002). Thus, diseases categorized by this reduction in air space diagnosed as interstitial lung disease (ILD) that were identified by Hammann and Rich in 1940s (Hammann et. al., 1944). The most common form
of ILD is IPF (Benjamin et. al., 2008). ILD/IPF is considered a model pathologic description of UIP where no inflammatory infiltration is required (Benjamin et. al., 2008). Definitely, the diagnosis of ILD/IPF requires a compatible clinical histology and surgical lung biopsy showing histological features of UIP (fibroblastic foci, temporal heterogeneity of fibrosis and mild inflammation) (ATS, 2001). On the other hand, NSIP, or non-specific interstitial pneumonia, diagnosed with more inflammatory cells (lymphocytes) and has better diagnosis then IPF (Watters et. al., 1987)

NSIP, which combines fibrosis and inflammation, differs in each pathology and prognosis from ILD/IPF. Patients with NSIP have a positive response toward corticoids and immunosuppressive drugs, better survival rate, well defined distinct histological prognosis with diffuse and temporally uniform pattern of lung injury combined with major inflammation and fibrosis loci, and absence of honeycombing (Travis et. al., 2000; Tsubamoto et. al., 2005). The American Thoracic Society/European Respiratory Society classification merges the histopathologic pattern and the clinical information of lung biopsy to introduce it in an ultimate clinicopathologic diagnosis (Table 3) (King et. al., 2004). Tables 3 summarize the key clinical and radiologist feature of ILP. Generally, IPF and NSIP are the most common and important type of ILP (King et. al., 2004).
<table>
<thead>
<tr>
<th></th>
<th>IPF/UIP</th>
<th>NSIP</th>
<th>COP</th>
<th>AIP</th>
<th>DIP/RB-ILD</th>
<th>LIP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration of Illness</strong></td>
<td>Chronic (&gt;12 months)</td>
<td>Subacute to chronic (months to years)</td>
<td>Subacute (&lt;3 months)</td>
<td>Abrupt (1 to 2 weeks)</td>
<td>Subacute (weeks to months); current or former smoker</td>
<td>Chronic (&gt;12 months); Women</td>
</tr>
<tr>
<td><strong>Frequency of Diagnosis</strong></td>
<td>47-64%</td>
<td>14-36%</td>
<td>4 to 12%</td>
<td>Rare (&lt;2%)</td>
<td>Diffuse; bilateral, ground glass opacities/progression to consolidation</td>
<td>Rare</td>
</tr>
<tr>
<td><strong>Chest x-ray</strong></td>
<td>Bilateral reticular opacities in lower zones; volume loss; honeycombing</td>
<td>Bilateral hazy and reticular opacity</td>
<td>Patchy bilateral consolidation</td>
<td>Ground glass opacities in the lower zones</td>
<td>Reticular opacities, nodules</td>
<td></td>
</tr>
<tr>
<td><strong>HRCT</strong></td>
<td>Peripheral, subpleural, basal predominance</td>
<td>Peripheral, subpleural, basal, symmetric</td>
<td>Subpleural/bronchiolar</td>
<td>Diffuse; bilateral, ground glass opacity in the middle and lower lung zones</td>
<td>DIP: Diffuse ground glass opacity; Centrilobular nodules; Patchy ground glass opacity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reticular opacities, honeycombing</td>
<td>Ground glass attenuation</td>
<td>Patchy consolidation and/or nodules</td>
<td>Ground glass opacities often with lobular sparing</td>
<td>Collagenous thickening; Septal and bronchovascular thickening</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Traction bronchiectasis/bronchiolectasis; Architectural distortion</td>
<td>Consolidation</td>
<td>Lower lobe volume loss</td>
<td>DIP: Diffuse ground glass opacity in the lower lobes; Centrilobular nodules; Patchy ground glass opacity</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pathology</strong></td>
<td>Focal ground glass</td>
<td>NSIP pattern</td>
<td>Organizing pneumonia pattern</td>
<td>Diffuse alveolar damage pattern</td>
<td>DIP pattern</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>Poor response to corticosteroid or cytotoxic agents</td>
<td>Corticosteroid responsiveness</td>
<td>Corticosteroid responsiveness</td>
<td>Mechanical ventilation; Effectiveness of corticosteroid unknown</td>
<td>RB-ILD pattern</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Corticosteroid responsiveness</td>
<td></td>
</tr>
<tr>
<td><strong>Prognosis</strong></td>
<td>50% to 70% mortality in 5 years</td>
<td>Unclear; &lt;10% mortality in 5 years</td>
<td>Rare deaths</td>
<td>60% mortality in &lt;6 months</td>
<td>5% mortality in 5 years</td>
<td>Not well defined</td>
</tr>
</tbody>
</table>

Table 2. Distinguishing clinical features of ILP (King et al., 2004).
3- Pathology

ILD/IPF has been known as an inflammatory disease with chronic alveolitis preceding fibrotic response (Keogh et. al., 1982). However, recent studies showed that inflammation is not the trigger for fibrosis in ILD/IPF (Selman et. al., 2001; Pardo et. al., 2002). Although, intra-alveolar macrophages and inflammatory cells accumulate in the lungs of patients with IPF histology, alveolitis is not a predictor of severity (Katzenstein et. al., 1998).

In addition, anti-inflammatory treatment with medications like corticosteroids are not effective in improving disease progression, suggesting that inflammation is not the root cause of ILD/IPF. In fact, lung injury followed by unsuccessful repair of alveolar epithelium in a chronic wound healing-like environment is thought to be an important pathogenesis of ILD/IPF (ATS 2000). These observations support the failure of immunosuppressive and anti-inflammatory treatments in ILD/IPF (Figure 1) (ATS 2000; Selman et. al., 2002; Fisher, 2006).
Figure 1, Hypothetical scheme of the main pathogenic events in ILD/IPF. Unknown insults provoke multiple microscopic foci of epithelial damage and stimulation. Activated alveolar epithelial cells release factors inducing fibroblast migration and proliferation and changes in cell phenotype. In the microenvironment of the lesion, myofibroblasts can induce epithelial cell apoptosis and basement membrane disruption, thus contributing to abnormal re-epithelialization and perpetuation of a vicious circle. Finally, fibroblasts/myofibroblasts secrete excessive amounts of extracellular matrix components and disequilibrium between MMPs and TIMPs means that matrix degradation does not occur. The final result is an aberrant remodeling of the lung parenchyma (Selman et. al., 2003).

4- Mechanism of wound healing in pulmonary fibrosis

While there is much speculation and hypothesis, the fundamental mechanisms underlying IPF is still unclear. What is known experimentally and pathologically is that following lung injury, a common fibrotic coagulant cascade of wound repair is activated and initiates a series of events including cellular recruitment, the exposure of matrix to cellular elements in the lung, and activation of several developmental and fibrogenic gene pathways. Given the temporal
heterogeneity seen in IPF (lung injury that is separated in time and location), there is likely repetitive lung injury. Repetitive injury and abnormal wound healing can lead to production of chemokines, fibrosis, growth factors and cellular recruitment. These events likely reflect an abnormal lung microenvironment seen in this lung disease.

Risk factors for ILD/IPF that cause fibrotic lung injuries include: cigarette smoking, exposure to certain prescribed drugs (such as antidepressants), chronic aspiration, environmental particles, allergens, radiation, infectious pathogens, and hereditary factors (Baumgartner et. al., 1997; Hubbard et. al., 1998; Tobin et. al., 1999; Marsh et. al., 1994; American Thoracic Society, 2000). These factors generate reactive oxidative molecules that can damage and activate local cells and extracellular matrix (ECM), which eventually injures the normal lung tissue causing disruption and mechanical damage to the architecture of the lung. This damage leads to inflammation that can contribute to cellular and tissue destruction (Kinnula et. al., 2003). Multiple and repetitive injuries damage the endothelial and alveolar epithelial layers (AEC) of the lung, leading to release of inflammatory mediators and factors such as thrombin (Pardo et. al., 2002). Thrombin can activate lung fibroblasts to proliferate and differentiate into collagen-producing α-SMA-myofibroblasts (Chambers et. al., 1998). Inhibition of thrombin decreases the accumulation of collagen in bleomycin-induced pulmonary fibrosis in mice (Howell et. al., 2002). In addition, alveolar epithelial cells (AECs) synthesize profibrotic, procoagulant molecules, growth factors and
multiple cytokines such as platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), tumor necrosis factor-alpha (TNF-α), connective tissue growth factor (CTGF), and endothelin-1 (ET-1), which provoke migration and proliferation of fibroblasts (Giaid et. al., 1993; Shi-wen et. al., 2004; Kapanci et. al., 1995, Pan et. al., 2001; Pardo et. al., 2002, Chambers et. al., 2003; Antoniades et. al., 1990).

Moreover, injury to lung endothelial and alveolar epithelial cells encourage platelet recruitment to the injured site, increasing vascular permeability, recruitment of leukocytes, increased MMP expression and collagen deposition and disruption of the extracellular matrix (ECM) barrier to enhance the extravasations of cells out and into the fibrotic lesion (Pardo et. al., 2006; Raghu et. al., 2006; Chambers et. al., 2003; Gabazza et. al., 1999; McKeown et. al., 2008). It is worth mentioning that the timing of the inflammatory response plays an important role in the progression of ILD/IPF. Early inflammatory response of the disease may promote wound healing and increase TGF-β and IL-13 production. However, in late responses, those after the first insult and wave of inflammatory cells, inflammatory cell recruitment may contribute in phagocytic macrophage recruitment, clearing fibroblast cell debris and inactivating and secreting T-helper 2 (Th2) and M2 macrophages that secrete IL-10 and activate the fibrogenic pathway (Salem et. al., 2001; Moodley et. al., 2003; Ohno et. al., 1992).
Indeed, the timing of inflammatory response to lung injury significantly influences healing. Inflammatory cells propagate inflammation by cytokines, chemokines and growth factor secretion. These proteins promote wound healing and pro-fibrotic activity through activation of certain genes (Fertin et al., 1991; Strutz et al., 2001). In addition, the timing of inflammatory response plays an important role in the progression of ILD/IPF, especially when early inflammatory response of the disease may promote wound healing and increase TGF-β and IL-13. However, in late responses, those inflammatory cells may contribute in phagocytes, clearing cell debris and inactivate fibroblast cellular proliferation through suppression of TGF-β which indeed, may lead to successful normal healing (Salem et al., 2001; Moodley et al., 2003; Ohno et al., 1992).

The final phase of wound healing involves a series of cellular re-organization of fibrin-rich scaffold formation, wound contraction and re-generation of the epithelial layer. This fibrin-rich scaffold formation is generated by ECM, platelets, macrophages and fibronectin-fibroblast (Muro et al., 2008; Hernnas et al., 1992). Besides fibronectin, many growth factors and TGF-β-activated fibroblasts are also involved in the healing process by inducing contractile and stress fibers that reduce the size of the wound (Singer et al., 1982).

Collagen deposition at the injury area depends on the quantity of ECM and the activated myofibroblast (Thannickal et al., 2006). For the resolution of normal wound healing, α-SMA-myofibroblasts undergo apoptosis to reduce the
inflammation and terminate collagen deposition (Fattman et. al., 2008; Gill et. al., 2008).

It is reported that the most primitive morphological changes in the lung related to progressive fibrosis is the presence of fibroblastic foci (Kuhn et. al., 1991; King et. al., 2001; Kazenstein et. al., 1998). At these fibroblastic foci, fibroblasts modify their phenotype from a migratory to a pro-fibrotic phenotype to release ECM components (Selman et. al., 2002).

5- Gene expression in ILD/IPF

ILD/IPF is a multifactorial disease where multiple genetic factors are involved. IPF is a pathological and clinical diagnosis that relies on a specific constellation of lung exam findings, radiographic findings, and the presence of subpleural fibrosis, fibroblastic foci and temporal heterogeneity on lung tissue examination (Marshall et. al., 2000). In fact, the pathological diagnosis of usual interstitial pneumonitis (UIP) is synonymous with ILD/IPF (Steele et. al., 2005). While little is known about the genesis of ILD/IPF, there is a unique form of familial ILD/IPF, suggesting a role of genetic factors in the disease (Uchiyama et. al., 1997., Marshall et. al., 2000; Peabody et. al., 1950; Bonnani et. al., 1965, Steele et. al., 2005; Thomas et. al., 2002; Javaheri et. al., 1980; Lee et. al., 2005). Many studies on familial IPF revealed important molecular signaling pathways found in ILD/IPF, although both ILD/IPFs (familiar and sporadic IPF) have similar clinical characteristics (Lawson et. al., 2004; Zorzetto et. al., 2003). Recent studies have
elucidated a genetic basis in a small number of sporadic cases of ILD/IPF, including some forms of familial IPF. Work from two independent laboratories identified mutations in telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) genes encoding telomerase components resulting in telomere shortening (Capkova et. al., 2007; Tsakiri et. al., 2007; Armanios et. al., 2007). However, mutations in TERT and TERC only account for up to 10% of familial IPF and fewer cases of sporadic diseases (Leon et. al., 2010; Tsakiri et. al., 2007).

Several published studies focus on analyzing pathological ILD/IPF lung tissue using mRNA gene expression profiling. These studies were done to identify the molecular pathways involved, monitor the transcriptional behavior of genes, understanding the responses of different cellular systems and underlying pathogenesis of ILD/IPF disease. These studies showed distinct gene expression patterns in ILD/IPF compared to unaffected/non-IPF lung tissue. In these reports, there were over 7,000 genes changed in ILD/IPF. These genes were clustered in groups according to their function, such as biological processes, cellular processes, and molecular processes (Figure 2). Many of them were significantly increased in fibrotic lungs and involved in the formation of extracellular matrix and smooth muscle actin (SMA) (Zuo et. al., 2002; Cojocaru et. al., 2001; Kaminski et. al., 2001).
Figure 2. The distribution of genes increased in idiopathic pulmonary fibrotic lungs.
Functional categories: (A) Biological process. (B) Cellular localization. (C) Molecular function. All enrichments are statistically significant (Fisher’s exact score, p < 0.03) Kaminski et al Proc Am Thorac Soc 3:339, 2001
Several genes such as connective tissue growth factor (CTGF), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), platelet-derived growth factor beta polypeptide (PDGF), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1 beta (IL-1β), granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF), collagen, and matrix metalloproteinase (MMPs) were highly expressed in ILD/IPF (Kaminski et. al., 1999; Zuo et. al., 2001; Kaminski et. al., 2006; Selman et. al., 2008; Simler et. al., 2004; Ramos et. al., 2010). However, there are no
reports of mutations in these genes or transcription factors regulating their expression in this patient population.

6- Treatment

ILD/IPF is the most widespread form of idiopathic interstitial lung disease with the lowest survival rate. It progressively increases in incident rate by aging, suggesting that ILD/IPF may be a disease of cellular injury with the inability to remodel the lung efficiently (a disease of cellular senescence). The incident rate is higher in men and in the elderly. Lack of information, understanding, and poor prognosis of the nature of the disease is a critical barrier to effective treatment and prevention strategies in ILD/IPF patients (Gribbin et. al., 2006). Treatment of ILD/IPF was dependent on the theory that inflammation leads to injury and fibrosis (Iwai et. al., 1994). To date, most treatment strategies focus on suppressing inflammation or inhibiting fibrosis rather than impacting the root cause of the disease. By understanding the underlying cause of the disease, we can intervene in meaningful ways for these patients.

For decades, the treatment options included corticosteroids, immunosuppressive/cytotoxic and anti-fibrotic agents, alone or in combination (Rudd et. al., 1981; Winterbauer et. al., 1978; Panos et. al.,1990; Johnston et. al.,1993; Johnson et. al.,1989; Douglas et. al.,1998; Raghu et. al.,1991; Rennard et. al.,1988; ATS 2000).
Glucocorticoids have been a traditional therapy in conjunction with azathioprine or cyclophosphamide to reduce inflammation in the parenchyma of the lung. In a double-blind, placebo-controlled trial in the treatment of ILD/IPF, 10-30% of ILD/IPF patients improved transiently (King et. al., 2003; ATS 2000). However, this regimen did not alleviate disease progression and is associated with significant side effects. Immunosuppressive or cytotoxic treatment (azathioprine or cyclophosphamide) is applied to patients with risk of corticosteroid complications, although no data exist to show an advantage of this treatment over corticosteroids. However, this approach is not effective, either. In addition to lacking clinical efficacy, cytotoxic therapy is potentially toxic (Baughman et. al., 1992; Hunninghake et. al., 1995). Other treatments such as colchicine and other agents that alter/inhibit collagen synthesis have been used as therapy for ILD/IPF. It is worth mentioning that these agents do not help in the long term analysis, and thus, are not currently used (Douglas et. al., 1998).

In summary, there is no evidence for therapeutic benefit for any of these therapeutic agents to improve the survival rate or the quality of ILD/IPF patient’s lives (Selman et. al., 2004; Maple et. al., 1996; Mason et. al., 1999). According to the present international guidelines of ATS/ERS, combined therapy of corticosteroid and azathioprine, or cyclophosphamide, can be used for a minimum of 6 months if no intolerable side effects occur. The therapy should be carried out only in patients who show objective improvement or stabilization of the condition continuously (ATS 2000). During therapy the patients should be
monitored carefully because of the possible and probable adverse effects which reflect the lack of effective therapy (ATS 2000). However, many experts do not pursue treatment with the diagnosis, and instead focus on early referral to a transplant center for evaluation and close monitoring of oxygen blood levels to ensure that secondary problems, like pulmonary hypertension (related to chronically low oxygen), is prevented. Despite this aggressive treatment, ILD/IPF is a progressive, irreversible, and lethal disease (Gross et. al., 2001; Selman et. al., 2001; DuBois et. al., 2001). Therapies with alternative approaches are needed to improve the prognosis of ILD/IPF by inhibiting fibroblast proliferation and ECM accumulation, protecting the epithelial layer from damages, and inducing myofibroblast apoptosis (Selman et. al., 2001; Fellrath et. al., 2003).

II. MicroRNA

MicroRNAs (miRNAs) are small non-coding RNAs (approximately 21-25 nucleotides (nt) long) that are evolutionarily-conserved and regulate fundamental cellular processes including cellular differentiation (Georgantas et. al., 2007; Hatfield et. al., 2008), cell cycle regulation (Lewis, 2005;), and metabolism (Poy et. al., 2004; Gauthier., 2006). It has been predicted that more than 30% of human genes are targeted by miRNAs (Ambros et. al., 2004; Lewis, 2005; Carleton et. al., 2007). The biogenesis of mature miRNAs is a multistep process involving several enzymes and proteins that begins in the nucleus and ends in
the cytoplasm and involving several enzymes and proteins (Lagos-quintana et. al., 2001).

1. miRNA Production

In the nucleus, miRNAs are transcribed as long, primary-miRNA by RNA polymerase II (Bartel et. al., 2004). The miRNA is then processed by a universal RNase III Endonuclease (RNASEN/Drosha) to ~70 nucleotide stem-loop precursor (pre)-miRNA with a 2-nt 3' overhang (Chen et. al., 2005; Bartel et. al., 2004). Then, Exportin-5/dsRNA binding protein will facilitate the exportation of pre-miRNA to the cytoplasm (Bohsack et. al., 2004; Gregory et. al., 2004; Lund et. al., 2004). In the cytoplasm, the endonucleases Drosha and Dicer further process the pre-miRNA to mature, functional 22-nt, single-stranded miRNA (Ambros et. al., 2004; Bartel et. al., 2004).

Dicer is a member of RNase III superfamily that binds to Drosha and further processes the pre-miRNA in the cytoplasm, to mature functional 22-nt single-stranded miRNA (Ambros et. al., 2004). The mature miRNA requires argonaute proteins to integrate into the RNA-induced silencing complex (RISC) to mediate post-transcriptional regulation. RISC complex is programmed by incorporation of mature miRNAs to repress the target mRNA at transcriptional and/or the translational level (Bernstein et. al., 2001) (Figure 3). The formation of miRNA-mRNA complex does not determine the fate and technique of mRNA repression. Instead, the fate of the target mRNA depends on the base pairing between the
mRNA 3'UTR and the miRNA seed sequence. The higher the complementary in mRNA:miRNA duplex, the more stable and efficient is the interaction (Lewis et. al., 2003; Brennecke et. al., 2005).

Notably, suppression of mRNA translation by miRNAs can go undetected by microarray analysis, as miRNA binding can also inhibit translation without reducing mRNA. Moreover, one miRNA is capable of regulating the expression of upwards of 100 genes (Lim ey al.,2005). Many databases have been established to predict the targets of the miRNA and vice versa.
2. Role of miRNA in heart and lung development

Many studies have shown the role and regulation of miRNAs in development in different organisms ranging from lower to higher living organisms. Importantly, recent studies by Mendell et. al., William et. al., and others illustrated the role of miRNAs in heart and lung development (Ventura et. al., 2008; Mendell et. al.,
2008; Harris et. al., 2006; Wang et. al., 2006; William et. al., 2007; Nana-sinkam et. al., 2009; Carraro et. al., 2009; Chen et. al., 2000) (Figure 4). These studies reveal that expression of many miRNAs (single and clustered) is changed during heart and lung development and disease. Deletion of miR-1-2 in mice interfered in the formation of ventricular septum formation to cause postnatal lethality (Zhao et. al., 2007). Furthermore, miR-17-92 deletion causes severe neonatal lethal lung hypoplasia resulting from diminished numbers of lung epithelial cells (Ventura et. al., 2008) while over-expression of the miR-17~92 cluster causes proliferation of epithelial cells, ultimately resulting in lung cancer development (Hayashita et. al., 2005).
Figure 4. Expression of miRNAs in lung development and disease. Shown are changes in miRNA expression identified in both human and murine models of lung development and disease. The developmental miRNAs indicated include those found to be differentially-expressed in human as well as miRNAs conserved in both human and mice. To date, all studies examining lung inflammation have only focused on miRNAs that increase in expression upon bacterial challenge. Several miRNAs that are highly expressed in fetal lung tissue appear to be re-expressed in response to smoke and lung carcinomas (shown in green). Notably, three members (miR-17-5p, miR-19b and miR-20a) of the miR-17~92 cluster are highly expressed in fetal lung. By contrast, several miRNAs found to be highly expressed in adult tissue are decreased in disease (shown in red). Figure from Nana-Sinkam et. al., American Review of Respiratory and Critical Care Medicine, 2009.

3. miRNA-17~92 Cluster

One of the most significantly-changed miRNAs expression in lung development and lung disease is mediated by the miR-17~92 cluster. This cluster encodes seven miRNAs (miR17-5p, miR-17-3p, miR-18a, miR-19a, miR-
19b-1 and miR-92a-1). These miRNAs are highly conserved among vertebrates and invertebrates and serve as a common miRNA oncomir-1 signature in several solid tumors (Volinia et. al., 2006; Lewis et. al., 2003). Although miR-17-5p and miR-17-3p are derived from the same precursor, 5′ miRNA (miR-17-5p) is the predominant form produced. miR-17-3p has the reverse complementary strand with unclear function. (Tanzer et. al., 2004; Landgraf et. al., 2007; O’Donnell et. al., 2005; Mourelatos et. al., 2002; Nana-Sinkam et. al., 2009). The miR-17~92 is a polycistronic cluster that has a single open reading frame 13 (C13orf25) located over ~800-nt within the third intron of chromosome 13 over ~800-nt on (13q31.3) in humans (Hayashita et. al., 2005; Lu et. al.,2005; O'Donnell et. al., 2005; Dews et. al., 2005; Diosdado et. al., 2009; Lewis et. al., 2004). Expression of the miR-17~92 cluster is regulated by the transcription factors c-MYC and E2F1 (O'Donnell et. al., 2005; Mendell, 2008; Dews et. al., 2006). This regulation adds complicity and involvement of the cluster in many c-MYC and E2F1 apoptotic/proliferative signaling pathways (Sylvestre et. al., 2007; Woods et. al., 2007; O'Donnell et. al., 2005). Interestingly, while the majority of miRNAs are decreased in cancer cells, the expression of the miR-17~92 cluster is increased, especially in lung cancer and B-cell lymphomas (Hayashita et. al., 2005; Rinaldi et. al., 2007; Diosdado et. al., 2009). It is reported that increased miR-17~92 expression allows for the decreased expression of the anti-angiogenic proteins including connective tissue growth factor (CTGF) and thrombospondin-1 (TSP-1) (Dews et. al., 2006).
In addition, this cluster is important in heart and lung development, B-cell development and lymphomagenesis (Egle et. al., 2004.; Ventura et. al., 2008), cell cycle regulation and tumorgenesis (He et. al., 2005), transcriptional regulation, and aging and senescence (Olive et. al., 2010; Rodier et. al., 2007; Lvanovska et. al., 2008; Campisi et. al., 2003; Mu et. al., 2009 Bartel et. al., 2004; Hornstein et. al., 2006; Bonauer et. al., 2009). This cluster also regulates cellular proliferation, angiogenesis (He et. al., 2005), and cell survival through the post-transcriptional repression of a number of target mRNAs (Figure 5).

Figure 5. The pleiotropic functions of miR-17~92 achieved by repressing specific targets. (Olive et. al., 2010).
In the lung, miR-17~92 is highly expressed in embryonic development and steadily declines throughout development and into adulthood (Ventura et. al., 2008). Mice lacking the miR-17~92 cluster exhibit an altered phenotype characterized by smaller size, lung hypoplasia, cardiac ventricular septal defects, and deficiencies in normal B-cell development (Ventura et. al., 2008) (Figure 6).
Figure 6. Characterization of miR-17–92-deficient mice (A) Macroscopic appearance of wild-type and miR-17–92-deficient embryos at various developmental stages. (B) Body mass of wild-type (WT), heterozygous (HET) and miR-17–92-null (KO) E18.5 embryos. The boxes indicate the 25th–75th percentile range. The horizontal lines represent the median, and the error bars indicate the lowest and highest values. P values were calculated using the two-tailed t test. (C) Macroscopic appearance of wild-type and miR-17–92-deficient lungs and hearts from E17.5 embryos. Notice the markedly hypoplastic lungs in miR-17–92 null embryos. (D) Hematoxylin-eosin staining of comparable transverse sections through the hearts of E18.5 wild-type and miR-17–92-null embryos. The arrow indicates a ventricular septal defect. (E) Flow cytometry plots of fetal liver cells from E18.5 wild-type and mutant embryos showing a marked reduction of B220+;CD43−;IgM− pre-B cells in mice lacking miR-17–92. The results are representative of three independent experiments. (F) Annexin-V staining showing increased apoptosis in miR-17–92-deficient fetal liver. B cell (blue) compared to wild-type control (red). The right panel shows the same analysis performed on B220-negative cells (Ventura et. al., Cell, 2008).
Lungs in these animals lack normal epithelial cells and because their terminal alveolar units are undeveloped, they die soon after birth of asphyxia (Ventura et al., 2008). Conversely, murine models of lung-specific miR-17~92 over-expression result in highly proliferative, undifferentiated lung epithelium (Lu et al., 2008) (Figure 7). These observations suggest that the cluster acts as key element in epithelial cell precursor production and implicates this cluster in lung development and epithelial cell repair.
Figure 7. Abnormal lung phenotype observed in miR-17~92 cluster transgenic embryos and newborn pups. (A) Schematic illustration of miR-17~92 cluster transgenic construct. The cluster contains all six miRNA precursors and 133 bp genomic flanking region at both ends. (B–I) H&E stained sections of left lobe from both the normal and transgenic lungs. (D, E, H, I) The enlarged pictures of panels B, C, F and G respectively. (J, K) miR-17-5p in situ hybridization of normal and transgenic lungs. (L) Microarray analysis shows that the expression levels of all six miRNAs within the cluster are increased in the transgenic lungs. Tested RNA was extracted from P0 lungs six transgenic and six normal pups from one litter of the transgenic mosaic founder. (B, C, F, G) Scale bar = 200 μm; (D, E, H, I) Scale bar = 100 μm; (J, K) Scale bar = 15 μm (Lue et. al., 2007)
A. miRNA-17~92 Paralogs.

The functional analysis of the miR-17~92 cluster is convoluted by the existence of two paralogs: miR-106a~363 and miR-106b~25. It is thought that all three miR-17 families have one origin and represent a series of tandem duplication and deletion events during vertebrate evolution (Tanzer et. al., 2004). miR-106a~363 is located on chromosome X in humans and mice and consists of six miRNAs, while the miR-106b~25 locus maps to intron 13th of DNA replication gene Mcm7 chromosome 7 in humans and chromosome 5 in mice, and consists of 3 miRNAs (Figure 8) (Ventura et. al., 2008; Carraro et. al., 2009). These three miRNA clusters have high sequence similarity and share overlapping sets of predicted targets (Lewis et. al., 2005; Sethupathy et. al., 2006). In 2008, Ventura showed that only the miR-17~92 cluster is required for normal development, and the other two clusters are dispensable (Ventura et. al., 2008).
Figure 8. Gene structure of human *miR-17~92* and its homologues. Primary transcript organization of human *miR-17~92* and its paralog, *miR-106a~363* and *miR-106b~25* clusters. The miRNAs encoded by the three clusters can be categorized into four separate miRNA families according to their seed sequences, including the *miR-17* family (*miR-17, miR-20a, miR-20b, miR-106a, miR-106b, and miR-93*), the *miR-18* family (*miR-18a and miR-18b*), the *miR-19* family (*miR-19a, miR-19b-1, and miR-19b-2*) and the *miR-92* family (*miR-92a-1, miR-92a-2, miR-383, and miR-25*) (Olive et. al., 2010).

**B- Regulation of *miR-17~92* Cluster**

The *miR-17~92* cluster has a promoter and an Open Reading frame (ORF) (Dews et. al., 2006; Sylvestre et. al., 2007). This promoter is regulated by the oncogene transcription factor C-myc in human B-cells and chronic leukemia cells (O'Donnell et. al., 2005; Venturini et. al., 2007). In neuroblastoma and medulloblastoma cells, *miR-17~92* cluster is regulated by c-MYC and n-MYC (Northolt et. al., 2009). In pulmonary hypertension, the inflammatory cytokines (*VEGF, STAT3* and *IL-6*) cause elevation of *miR-17~92* cluster expression and rescue of angiogenesis (Suarez et. al., 2008; Brock et. al., 2009).
Drosha and Dicer play crucial roles in processing and maturing the miR-17~92 cluster. This processing step involves many RNA binding proteins that selectively bind certain miRNAs and manipulate their expression. hnRNP-A1 RNA binding protein binds to miR-18a and enhances its maturation and expression without affecting the expression of other neighboring miRNAs in the cluster (Thomson et. al., 2006; Newman et. al., 2008; Davis et. al., 2008).

Importantly, c-MYC is not the only regulator of miR-17~92 cluster expression. Cell cycle regulators such as E2Fs and cyclin-D1 play important roles in regulating the expression of the cluster in many cancer cells.

II. EPIGENETIC REGULATION

The term “epigenetic” involves two major concepts: I) the study of heritable processes in gene expression without a change in the genomic DNA sequence; and II) the study of heritable developmental changes within an organism (Bird, 2007). These changes are critical for determining transcriptional outcome affecting the development and cell differentiation of various cell types. Environmental parameters and/or aging can affect the way of epigenetic modification on the cell cycle (Esteller et. al., 2006). Epigenetic modulation plays an essential role in development, embryogenesis, carcinogenesis, and other disease processes (Klose et. al., 2006; Baylin et. al., 2006; Jones et. al., 2002). The study of epigenetics is a rapidly maturing field that is based on the
realization that complex biological pathways which regulate DNA elements, is critically affected by the environment and behaviors of the host.

Cells have evolved machinery to manage the tight regulation of gene expression and other nuclear process such as replication, DNA repair, and recombination (Muhonen et. al., 2009). One of the principal challenges of any organism is the packaging of its genome. Genomic DNA is considering very long (~2m) that needs to be compressed and packaged efficiently in the nucleus. Eukaryotic genomes are packaged into two highly-organized chromatin structures: highly compact DNA regions where DNA transcription is silent, including centromeres and telomeres (heterochromatin), or less compact DNA structures of active transcription (euchromatin) (Muhonen et. al., 2009; Elgin et. al., 2003; Dimitri et. al., 2005). These two chromatin states are regulated by epigenetic DNA methylation and histone modifications that control the expression of different genes without mutation or change in the DNA sequence (Figure 9).

By packaging the DNA into chromatin, it becomes inaccessible to cellular factors which inhibit DNA-based processes. To conquer this limitation, cells have developed machinery to alter the chromatin structure and maintain the genomic flexibility. At this time, we know four mechanisms the cell has developed to modulate chromatin structure: 1) DNA methylation at CpG-rich sequences; 2) histone post-translational modification; 3) ATP-dependant chromatin remodeling; and 4) RNA regulatory systems like miRNAs.
Figure 9. Schematic figure of the epigenetic regulation mechanism. DNA methylase catalyze the transfer of a methyl group to the cytosine residues in CpG dinucleotide sequences. (A). Methylation of the CpG islands in the gene promoter region inhibits gene expression. (B). Histone acetylation plays an important role in the regulation of gene expression. Hyperacetylated chromatin is transcriptionally-active whereas hypoacetylated chromatin is silent. Methyl-CpG-binding proteins interact with histone deacetylase causing gene silencing (C) (Muhonen et. al., 2009).
1- DNA Methylation

DNA methylation is an important epigenetic process absent in lower eukaryotic organisms, indicating that this modification evolved later than histone acetylation and methylation (Robertson et al., 2002). It is the most characterized epigenetic alteration in humans. DNA methylation represses the transposable elements and repetitive sequences to mediate long-term silencing and genomic stability (Jones et al., 2002; Bird 2002).

DNA methylation is carried by a class of enzymes referred to as DNA methyltransferases (DNMTs) which reversibly transfer a methyl group from a methyl donor, S-adenosyl-methionine (SAM), predominantly to C-5 of the cytosine ring, in the cytosine-guanine dinucleotide rich region (CpG island) to generate 5-methyl-cytosine (me-C) (Bird 2002). In normal DNA, CpG islands are not methylated and are available for active transcription (Clark et al., 2002).

The CpG dinucleotide occurs at lower frequency in mammals (less than 4%) of the genome, existing in approximately 40-50% of the promoter region of genes and first exons (Gardiner et al., 1987). CpG islands were initially defined as 50% CG-rich area with five times more occurrence of CG dinucleotides than the rest of the genome sequence (Herman et al., 2003; Baylin et al., 2005; Gardiner et al., 1987).

To avoid confusion between CpG islands and other CG-rich areas, such as Alu-repetitive elements, the definition of a CpG island has been modified with stringent criteria. The most important criteria is that the CG content of the CpG
island should be greater than 500 base pairs, with CG content equal or greater than 55%, and the ratio of observed CpG/expected CpG is at least 0.65 (Takai et. al., 2002). In cancer, the promoter of tumor suppressor genes (TSG) is usually hypermethylated, which explains the silencing behavior of many TSG (Greger et. al., 1989).

Methylation of gene promoter regions correlates with gene silencing through the recruitment of transcriptional repressors (Bird et. al., 2002; Jones et. al., 2002). In the mass of genomic DNA, about 80% of CpG dinucleotides that are not associated with CpG islands are heavily methylated. Conversely, the dinucleotides in the CpG islands comprise 1% of the genome and are generally found with gene promoters are usually unmethylated to allow gene expression (Bird et. al., 2002). A number of methyl binding proteins (MBP) are known to associate with methylated CpG residues and recruit transcriptional repressors like mSIN3/HDAC (Jones et. al., 2002).

The pattern of DNA methylation can be classified into two groups according to the amount of DNA methylation: 1) DNA global hypomethylation and 2) CpG island hypermethylation. In cancer, tumor cell methylation is very low compared to normal cellular methylation (Feinberg et. al., 1983). This repression of DNA methylation correlates to intronic and exonic demethylation to allow another copy of mRNA to be transcribed (Feinberg et. al., 2004). This dominant hypomethylation phenomenon in tumors is increased with methylation of the
promoter region, according to the Multistep Molecular Carcinogenesis theory (Esteller, 2008).

DNA CpG island hypermethylation is considered a poor prognostic and diagnostic factor in cancer (Esteller, 2008). This makes it an attractive target for new therapeutic agents such as DNA methylation inhibitors in cancer. Currently, two DNA methylation inhibitors have been approved for clinical treatment. 5-aza-cytidine (Vidaza) is clinically used for leukemia (chronic myelomonocytic) and 5-aza-deoxycytidine (decitabine) is approved for clinical use in myelodysplastic syndromes (Muller et. al., 2006; Mack et. al., 2006; Oki. et. al., 2007).

A. DNA methyl transferases (DNMTs)

DNA methyl transferase enzymes (DNMTs) can catalyze the transfer of methyl groups from a methyl donor, SAM, to cytosine residues. In mammals, Five DNMTs are identified: \textit{DNMT-1}, \textit{DNMT-2}, \textit{DNMT-3A}, \textit{DNMT-3B}, and \textit{DNMT-3L} (Table 2). DNMTs contain an N-terminal regulatory domain that includes nuclear localization signal, a C-terminal catalytic domain, and a central linker domain (Cairns et. al., 2006; Kim et. al., 2002).

**DNMT-1** is the most abundant and largest DNA methyltransferase (184 kDa). It is the first purified and cloned DNA methyltransferase that preferentially methylates hemi-methylated DNA (Kim et. al., 2002). It is not responsible for gene hypermethylation alone. Both **DNMT-3A** and **DNMT-3B** cooperate in this process (Rhee et. al., 2002). **DNMT-1** has higher affinity for hemi-methylated
CpG dinucleotide strands (one strand is methylated). *DNMT-1* has diffuse nuclear localization in G1 cells and during S phase in the cell cycle. It is located at the replication fork to methylate the newly-synthesized DNA stands and maintain parental DNA methylation patterns (Lei et. al., 1996; Leonhardt et. al., 1992; Robertson et. al., 2000). Homozygous deletion of mouse *DNMT-1* causes embryonic lethality (Lie et. al., 1996; Lie et. al., 1993) severe demethylation of all sequences examined, and inactivation of X-chromosome caused by up-regulation of Xist gene (Li et. al., 1992; Lie et. al., 1996; Beard et. al., 1995). Moreover, *DNMT1-/-* embryonic stem cells undergo apoptosis even when they are provided with nutrients and differentiation factors. These results confirm that *DNMT-1* is the major and critical DNA methyltransferase in higher organisms (Lie et. al., 1996; Beard et. al., 1995).

*DNMT-2* is a much smaller molecule (45 kDa), and is the first DNMT to be identified to methylate tRNA in vertebrates (Coll et. al., 2006; Kim et. al., 2002). *DNMT-2* lacks N-terminal and central linker domains and has no effect on growth since homozygous null *DNMT-2-/-* mice are viable and normal (Okano et. al., 1998; Yoder et. al., 1998). Although *DNMT-2* contains a conserved catalytic domain, it lacks DNA methyltransferase activity (Okano et. al., 1998). While the role of DNMT-2 is not discovered yet in any species, it is proposed to have a role in centromere functioning (Yoder et. al., 1997).

*DNMT-3A* and *DNMT-3B* are intermediate in size (100-130 kDa). They are *de novo* methyltransferases that mediate establishment of new DNA methylation
patterns, while *DNMT-1* is the maintenance methylase that preserves the methylation pattern through cell division established by *de novo* methyltransferases. Inactivation of *DNMT-3A* and *DNMT-3B* genes blocks *de novo* methylation in mouse embryonic stems cells and early embryos, but does not affect the imprinted methylation maintenance (Lei et al., 1996; Sinkkonen et al., 2008). This indicates that both *DNMT-3A* and *DNMT-3B* function is to regulate *de novo* methyltransferase activity rather than maintenance activity (Okano et al., 1999).

Moreover, murine *DNMT-3A* and *DNMT-3B* have no preference for hemimethylated versus unmethylated oligonucleotides *in vitro* (Okano et al., 1998). Consequently, each *DNMT* is either *de novo* or maintenance methyl transferase (Bestor et al., 2000). *DNMT-3A* is ubiquitously expressed while *DNMT-3B* is predominantly expressed in the testis, thyroid, and bone marrow. *DNMT-3A* deficiency in mice is lethal in early adulthood with signs of aganglionic megacolon and loss of germ cells in males, while *DNMT-3B* knockout mice are not viable (Haigis et al., 2006) (Table 2).
CHAPTER TWO: MATERIALS AND METHODS

In this chapter, the materials and methods that were used throughout this study is included. Sometimes the method to isolate DNA or RNA differs depending on the nature of the sample.

1- Reagents

Media and cell culture supplements were purchased from Invitrogen (Carlsbad, CA) unless specified. FBS was obtained from Atlanta Biologicals (Lawrenceville, GA). If otherwise indicated, molecular biology reagents for RNA isolation and cDNA generation for mRNA expression were purchased from Invitrogen (Carlsbad, CA). Reagents for miRNA analysis by PCR were obtained from Applied Biosystems (Foster City, CA). Antibodies for Western blot analysis were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents including 5'-aza-2'-deoxycytidine (5-Aza) were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

2- Pathway analysis and prediction

Predicted miRNA targets were resolute by using the miRanda algorithm (http://microrna.sanger.ac.uk/targets/v5/) (version 5.1), Pictar (http://pictar.mdc-
miRbase (http://www.mirbase.org/search.shtml), miRNA map (http://mirnamap.mbc.nctu.edu.tw/), Diana-microTest (http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi/) and TargetScan v4.2 (http://www.targetscan.org/) databases. Common predicted miRNAs from each database were subjected to analysis and only common miRNA of each gene were selected to be tested.

3- Cell Culture techniques

Normal adult human lung fibroblasts (CCL-201 and CCL-204) and fibroblasts isolated from the lungs of patients with IPF (CCL-191 and CCL-134) were obtained from American Type Culture Collection (ATCC) (Rockville, MD). Normal lung fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 1% Penicillin/Streptomycin (100 U/100 mg/ml), and 1% sodium pyruvate. IPF lung fibroblasts were cultured in Ham's F12K medium containing 15% FBS and 1% Penicillin/Streptomycin. Cells were incubated at 37°C at 5% CO₂. Cells were cultured for periods less than 5 weeks.

4- Primary tissue samples

Identified tissue samples were acquired through the Lung Tissue Research Consortium (LTRC #07-99-0006), Lifeline of Ohio and the Cooperative Human Tissue Network (CHTN). The use of these samples was approved under IRB protocol# 2007H0002. IPF patient samples were obtained from the Lung Tissue
Research Consortium (www.ltrcpublic.org). Samples obtained from patients with IPF were segregated into three FVC groups: ILD-1 (<50% FVC), ILD-2 (50-80% FVC), or ILD-3 (>80% FVC, least severe breathing impairment). Control samples consisted of tissue rejected for lung transplantation and control normal adjacent tissue from lung biopsies from Lifeline of Ohio and the CHTN, respectively.

5- Hierarchical cluster analysis

Identical samples analyzed for mRNA expression were analyzed for miRNA expression by qRT-PCR. Correction of the raw PCR cycle threshold (CT) scores included normalization by the method of Vandesompele (Vandesompele et. al., 2002). In this study, Vandesompele normalization method was implemented using the SLqPCR Bioconductor library (Kohl, 2007). Color key: dark green, highest expression; dark red, lowest expression.

6- Microarray data analysis and network construction

Affymetrix array data was summarized, normalized, and transformed in log2-scale by the GCRMA method (Wu and Irizarry 2004). For the Agilent miRNA array, a consolidated intensity value for each probe was obtained, quantified, normalized (Bolstad et. al., 2003) and log2-transformed. Probe sets were identified as present or absent by applying a Gaussian mixture model. We used only these present mRNA probe sets for subsequent statistical analysis, but for
miRNA data, all probes were used. A detailed description of the approach is in supplemental information.

7-In situ hybridization for miR-19b and miR-20a and immunohistochemistry

Frozen lung tissue was thawed slowly over 48 hours then fixed in 10% buffered formalin for 8-15 hours. The tissue was washed with 70% ethanol followed by washing 3 times in PBS at RT then the tissue was paraffin-embedded (Ohio State University Pathology Services) and cut to 4 micron sections. In collaboration with Dr. Gerald Nuovo (The Ohio State University), lung tissue sections were examined for the expression of miR-19b, and -20a by in situ hybridization using 250 pmol of digoxigenin 5’-labeled probe corresponding to miR-19b and miR-20a. The probes with the first 6-nt starting at the 5’ end LNA modified (TCAGTTTTGATGGATTTGCACA), miR-19b ultramer (unlabeled) GACCCCTTAATGCGTCTAAGACCCCTTAATGCGTCTAAGCAGCCCTTAATGCGTCTAA and miR-19b ultramer (unlabeled) (GACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAGACCCCTTAATGCGTCTAAGACCCCTTAATGCGTCTAAA GACCCCTTAATGCGTCTAAGACCCCTTAATGCGTCTAAGACCCCTTAATGCGTCTAAGACCCCTTAATGCGTCTAAA), as previously described (Crawford et. al., 2008). As a control, miR-let-7c was used as a positive control and scrambled miRNA probes as a negative control. Immunohistochemical techniques were employed to identify which cell types within the lung tissue that expressed the miRNAs and their targeted mRNA genes using certain antibodies.
against the gene of interest. From digital images, the immunostaining was quantified using Adobe Photoshop CS2 software and histogram analysis.

8- RNA isolation from human lung samples

Total RNA was isolated from human lung samples by homogenization using Soft Tissue Omni Homogenizer Tips (Omni International; Marietta, GA) and in Trizol® Reagent per the manufacturer’s instructions. Total RNA was extracted from cells using TRIzol reagent, as previously described (Wang et. al., 2007). RNA was isolated by Trizol extraction and precipitation at -20°C overnight to increase the yield of small RNAs, as previously described (Piper Hunter et. al., 2008). RNA integrity was confirmed by gel electrophoresis using the FlashGel® RNA cassette system (Lonza; Rockland, ME).

9- RNA Extraction

RNA was prepared from ATTC human lung fibroblast cell lines using Trizol reagent (Invitrogen, Inc., catalog no. 15506-018). Cells from approximately 90% confluent plate were collected, washed with 5 ml 1X PBS twice, and then 1 ml Trizol reagent was added. Next, 200µl of chloroform was added and then was mixed by vigorously shaking of the tube and incubating on ice for 10 minutes. Then, the sample was centrifuged at 13,000 rpm for 15 minutes at 4°C and the aqueous layer transferred to a fresh tube. RNA was precipitated by adding 500µl of isopropanol, mixed, and then 1µl of glycogen was added followed by
incubation at -20°C at least overnight. RNA was then collected by centrifugation of the samples at 13,000 rpm for 15 minutes at 4°C, the pellet was washed twice with 70% ethanol, and the RNA pellet air dried the at room temperature. Finally, RNA was resuspended in 15-30 µl of DEPC-treated water and stored at -80°C. The concentration was measured using a Nano-drop machine.

10- Transient Transfection

The day prior to transient transfection, fibroblasts were plated in six-well plates at a density of 0.5x10^6 to achieve 60-70% confluence. On the day of transfection, the cells were washed once in 1x PBS and fresh media was added to the cells then transfected using the siPORT NeoFX transfection reagent (Ambion, Foster City, CA) and either 0.5 or 1.0 µg of miR-17–92 in pcDNA3.1 expression vector (generously provided by Dr. Joshua Mendel, Johns Hopkins University, Baltimore, MD). Cells transfected with empty vector (pcDNA3.1) served as a control. Samples transfected in the absence of plasmid DNA served as a mock control. Cells were incubated for 24 hours then RNA was isolated. The samples were subjected to miRNA expression analysis as well as quantification of gene and protein expression.
11- Transient Transfection of miR-19b and miR-20a

The day prior to transfection, cells were plated in six-well plates at a density of 2.4x10^5 per well. The day of transfection, 9µl siPORT NeoFX Transfection Agent (Ambion, Foster City, CA) was diluted in 300µl medium in a sterile tube. After 10 minutes incubation at RT, 10 µM pre-miR precursor was added to the solution and incubate for 10 minutes at RT. Negative pre-miR and has-miR-1 precursor as a positive control was used at the same concentration as experimental miRNA. During the incubation time, the cells had 2.4ml of fresh media. Finally, transfection solution was added and incubated at 37°C for 24 hours. The Pre-miR™ miRNA Precursor Starter Kit Part Number AM1540 from Ambion was used.

12- Quantitative Real-Time PCR Analyses

Expression of mature miRNA Homo sapiens hsa-miR-17, has-miR-18a, has-miR-19a, has-miR-19b, has-miR-20a and has-miR-92a were analyzed by TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA). RNA (100 ng) was converted to cDNA by priming with looped primers specific for each miRNA according to manufacturers’ instructions using an Applied Biosystems, ABI 7900HT real-time PCR System. Primers to the internal controls, small nucleolar (sno) RNA U38B, snoRNU43, small nuclear (sn) RNA U6, and as well as hsa-miR-191 were used. For quantitative (q)RT-PCR expression, cDNA was synthesized from 5 µg RNA by oligo-dT primer and superscript II (Invitrogen,
Carlsbad, CA) then amplification was performed with the SYBR green-based detection system (Applied Biosystems, Foster City, CA). Commercial primer sets for DNMT-1, DNMT-3a, DNMT-3b, VEGF, SMA, CTGF, ELK-3, Collagen (Col)-1a, Col-3a, Col-13a, c-Myc, TSP-1, MMP7, MMP-9, CCL-2, and GAPDH were obtained from SABiosciences (Frederick, MD). Real-time PCR was performed using standard conditions. For normalization of expression levels, GAPDH and adenyl cyclase associated protein (CAP) primers were used. Human CAP-1 F 5’-ATTCCCTGGATTGTGAAATAGTC-3’ and human CAP-1R 5’-ATTAAAGTCACCCTTCTGTAG-3’ were purchased from Invitrogen. All PCR expressions were quantified using 2^ΔCt method (Piper Hunter et. al., 2008).

13- Western blot analysis

Protein analysis was performed by western blotting. Briefly, proteins were isolated from frozen tissue using freeze fracture techniques in cell lysis buffer (Cell Signaling Biotechnology, Danvers, MA) containing protease inhibitor cocktail III (EMD Calbiochem, Gibbstown, NJ). Samples were incubated for 15 minutes on ice then centrifuged to remove the insoluble fraction. The protein concentration was determined by the BCA protein assay (Bio-Rad, Hercules, CA). Cell lysates were separated by SDS-PAGE, transferred onto nitrocellulose membranes and subjected to Western blotting with the indicated antibodies. The immunoblotted proteins were detected by ECL reagent (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The ECL signal was quantified using the
Quantity One densitometry program (BioRad, Hercules, CA). Protein expression was normalized to either β-actin or total protein, respectively. Data are expressed as fold-change of stimulated samples over non-stimulated samples.

14- Senescence-Associated β-Galactosidase Staining

48 hours after transfection, IPF fibroblast cells were fixed and the senescence status was demonstrated by in situ staining for senescence associated β-galactosidase (SA- β-gal). Cells grown on 6-well tissue culture plate, washed 2X with 1x PBS then the cells were fixed by adding 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 minutes followed by another rinse with 1X PBS. Cells were then incubated in β-galactosidase staining solution (150 mmol/L NaCl, 12 mmol/L sodium phosphate pH 6.0, 5 mmol/L potassium ferrocyanide, 40 mmol/L citric acid, 5 mmol/L, potassium ferricyanide, 2 mmol/L MgCl₂, containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β –D-galactoside [X-gal]) for 24 hours at 37°C. Cells were washed with PBS to end the reaction. Statistical analysis was performed. At the same time, DAPI staining was used for cell counting and at least 400-500 cells were counted per experiment.

15- Cell proliferation assay

The growth rate of transfected lung fibroblasts with miR-17~92 cluster was compared to different a experimental control, untreated cells, mock and empty transfected cells by MTT assay.
The cells were cultured at a density of 4000-5000 cells/well in 24-well plates. Cell proliferation was monitored by MTT assay from day 0 (1 day after plating) to 48 hours. 300 µl of MTT reagent were added to each well and incubated at 37°C for 2 hours. The supernatant was removed and replaced with 400µl of dimethylsulphoxide solution/well and incubated at RT for 10 minutes. By using an enzyme–linked immunosorbent assay plate reader at 570nm, the samples were quantified. The assay was performed in triplicate and repeated 3 times.

16- DNA isolation

DNA was isolated from lung fibroblasts using PerfectPure DNA Cultured Cell Kit according to manufacturer’s instructions (5 Prime, Inc., Gaithersburg, MD). The QIAamp DNA Mini Kit was used to extract DNA from human lung samples (Qiagen, Valencia, CA).

17- Examination of DNA methylation patterns

To detect DNA methylation of the CpG Island in the promoter of the miR-17~92 cluster, the Methyl-Profiler™ DNA Methylation qRT-PCR Primer Assays (Cat# MePH90170-1A, SAbiosciences, Frederick, MD) was used. According to the manufacturer instructions, the samples were subjected to methylation sensitive and insensitive restriction enzyme digestions. An additional reaction containing both enzymes was performed. As a control, a reaction in the absence of enzymes was done. The reactions were subjected to PCR amplification using
specific primer pairs to the human miR-17~92 CpG Island. The resulting amplification patterns indicate the extent of methylation across that region. To determine the status of DNA methylation of miR-17~92 cluster CpG Island, the ΔCt raw threshold cycle value data was automatically performed using the Excel-based template supplied with the kit. This excel template normalizes the Ct values of both digests with the mock digestion values to calculate and report the percentage of the queried DNA that is hypermethylated, intermediately methylated, and unmethylated.

18- Inhibition of DNA methylation with 5’-aza-2’-deoxycytidine (5-Aza)

5’-aza-2’-deoxycytidine (Sigma Aldrich, St. Louis, MO) was a dissolved in PBS and stored at −80°C. IPF lung fibroblasts were seeded at 4-6 x10⁵ in a 100 mm dish then they were treated for 24-72 hours with 0.5 - 2.0 µM of 5’-aza-2’-deoxycytidine. For gene expression studies, 0.5 µM for 24 hours was chosen based on cell viability. Following incubation, RNA was extracted and expression of miR-17~92 cluster microRNA, DNMT-1, DNMT-3a, DNMT-3b, CTGF, VEGF, SMA, COL13A, COL1A, and COL3a were tested using RT-PCR method as mentioned above. The 5’-aza-2’-deoxycytidine solution was tested for contaminating endotoxin. As measured by Pyrogent Plus Gel Clot LAL Assay (Lonza Walkersville, Walersville, MD), levels were less than 0.06 EU/ml.
19- Luciferase Reporter Experiments

The 3'UTR for *DNMT-1* was amplified by PCR from genomic DNA and cloned into the pGL3 vector (Promega) and generously provided by Dr. F. Muller (The Ohio State University). Notably, the miRNA recognition sites overlap for *miR-17, miR-20a, miR-19b,* and *miR-92a.* Thus, a mutant 3' UTR lacking the recognition site for these four *miRNAs* was generated by deleting 13-nt. (5'-AUUGUGCAGUACUUUGUGCA-3') was done using the following primer set; FW:5'GTGATCAAATTGTGCTTCTGGATTTTA-3' RV:5'-CTTTTAAAATCCAGAACACAATTTGA-3' and QuikChange XL-site directed Mutagenesis Kit (Stratagene, Santa Clara, CA). Wild type (WT) and mutant inserts were confirmed by sequencing. Briefly, the HEK 293 cell line was co-transfected in six-well plates by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with 0.4 \( \mu \)g of the firefly luciferase report vector and 0.08 \( \mu \)g of the control vector containing Renilla luciferase, pRL-TK (Promega, Madison, WI) for normalization. For each well, 10 nM concentrations of each *miRNA* (Ambion) were used. Firefly and Renilla Luciferase activities were measured consecutively by using the dual Luciferase assays (Promega) 24 hours after transfection. Experiments were performed in duplicates in four independent experiments.
18- Inhibition of DNA methylation with 5’-aza-2’-deoxycytidine

The 5’-aza-2’-deoxycytidine was dissolved in PBS and stored at −80°C. Human IPF lung fibroblasts were seeded at 4-6 x10^5 in a 100 mm dish then they were treated for 24-72 hours with 0.5 - 2.0µM of 5’-aza-2’-deoxycytidine. For gene expression studies, cells were treated with 0.5 µM 5’-aza-2’-deoxycytidine for 24 hours to maintain cell viability as measured by trypan blue exclusion and prevent nonspecific toxicity. Following incubation, RNA was extracted and expression of miR-17~92 cluster, DNMT-1, DNMT-3a, and DNMT-3b, as well as the indicated fibrotic genes were examined by qRT-PCR method as mentioned above. The 5’-aza-2’-deoxycytidine solution was tested for contaminating endotoxin by measuring with Pyrogent Plus Gel Clot LAL Assay (Lonza, Walkersville, MD). Levels were confirmed to be less than 0.06 EU/ml.

21- Mice and bleomycin administration

C57/BL6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experimental procedures were approved by the institutional review committee and the animals were handled in accordance with their guidelines. Mice underwent intra-peritoneal injection with 0.035U bleomycin/g or vehicle control (PBS) twice weekly for 4 weeks, as previously described (Baran et. al., 2007). One week following the last injection, mice were sacrificed and the lungs were insufflated at 20 cm pressure. The left lobe of the lung was placed in 10% formalin for immunohistochemical processing, and the right lobes were snap
frozen in liquid nitrogen for DNA and RNA processing. After paraffin-embedding, 10 µm sections were cut and stained with trichrome and H&E via the Pathology Core at The Ohio State University. Five images along the periphery were photographed using an Olympus microscope (Center Valley, PA) with a digital camera. Total pixels were counted per stain using Adobe Photoshop CS2 software and histogram analysis (San Jose, CA).

22- Treatment of mice with 5'-aza-2'-deoxycytidine

Because alterations induced by 5'-aza-2'-deoxycytidine can be repaired (Kelly et. al., 2003), a dose regimen of 2 times per week at 0.156 mg per kg body weight was given via intrapritoneal (i.p.) injection. This regimen was used to ensure continual incorporation of the drug into DNA while limiting toxicity to the mice based on an experimental dose curve (data not shown). Mice were also treated in combination with bleomycin and 5'-aza-2'-deoxycytidine. Mice were injected on alternating days. Bleomycin-treated mice received 5'-aza-2'-deoxycytidine two weeks after bleomycin or for the entire four week bleomycin regimen. Since inflammation and fibrosis begins after two weeks of bleomycin injections, this treatment was chosen to target the lung during the fibrotic process. PBS treated mice served as a control, as well as bleomycin only treated mice.
CHAPTER THREE: EXPRESSION OF \textit{miR-17-92} CLUSTER IN HUMAN LUNG IPF TISSUES

1- Introduction

Interstitial lung disease (ILD)/Idiopathic pulmonary fibrosis (IPF) is a chronic progressive interstitial pneumonia with unknown etiology. The first pathological classification of idiopathic interstitial disease was done by Liebow and Carrington in 1969 and they classified it into five groups; usual interstitial pneumonia (UIP), lymphoid interstitial pneumonia (LIP), giant cell interstitial pneumonia (GIP) bronchiolitis obliterans with interstitial pneumonia (BIP), and desquamative interstitial pneumonia (DIP) (Turner-Warwick et. al., 1980; Tukiainen et. al., 1983). Alternative classification systems for idiopathic interstitial pneumonias (IIP) exist, but these systems still have confusion in the terminology and diagnostic criteria due to overlapping, common symptoms, histological diagnosis and disease course (Willis et. al., 2005; Selman et. al., 2006). Thus, the need for precise and accurate standards of classification is necessary which was provided by collaborative efforts of the American Thoracic Society (ATS), European Respiratory Society (ERS), and American College of Chest Physicians (ACCP) (ATS 2002). These position statements define clinical, pathologic and radiologic criteria for diagnosis and classification of IIPs. This approach divides IIPs into IPF/usual interstitial pneumonia (UIP), desquamative interstitial
pneumonia (DIP), respiratory bronchiolitis with interstitial lung disease (RBILD), AIP, cryptogenic organizing pneumonia (COP), nonspecific interstitial pneumonia (NSIP), and lymphocytic interstitial pneumonia (LIP). ILD/IPF represents approximately 60% of all IIPs (Bjoraker et al., 1998; Thomeer et al., 2001, Gross et al., 2001).

Many researchers and clinicians focus on the pathogenesis of ILD/IPF in the hopes of designing effective and targeted therapies. For example, gene array studies of lung tissue from patients with ILD/IPF demonstrate the activation of fibrotic, developmental and inflammatory mRNA pathways. While no single gene is identified causing this disorder, experts speculate that genetically susceptible individuals suffer environmental insult(s) to their lungs resulting in lung injury and fibrosis.

Recent studies reveal that genetic change can be regulated at a post-transcriptional level without changes in DNA sequence by small non-coding RNAs, referred to as microRNAs (miRNAs). These miRNAs are 22-25 nucleotides in length and have the same processing machinery of siRNA. miRNAs are endogenous in the cell and a single miRNA can target up to hundreds of genes. miRNA binding to a target mRNA depends on 2-8 nucleotides on the 5’ end of the miRNA (seed region). This region is conserved through species and when there is complementarily with nucleotides the 3’UTR of a target mRNA where binding occurs and can either cause rapid degradation
of the mRNA or inhibition of protein translation (Bartel et. al., 2004; Lewis et. al., 2003; Brennecke et. al., 2005; Maziere et. al., 2007; Bartel et. al., 2004).

With the ability to bind even without perfect complementarily, miRNA have enormous capacity to impact gene regulation and this property distinguishes miRNA from siRNA, which have single targets (Lewis et. al., 2003; Kerk et. al., 2005). As a result, each miRNA is capable of regulating 60% of human protein coding genes (Gangaraju et. al., 2009; Papagiannakopoulos et. al., 2009; Friedman et. al., 2009).

Genes found in the lungs of patients with ILD/IPF are involved in crucial cellular pathways such as cell cycle regulation, metabolism, disease progression, angiogenesis, fibrosis, and embryonic development (Hagen et. al., 2007; Chen et. al., 2005; Sassen et. al., 2008; Carleton et. al., 2007; Hatfield et. al., 2008). In addition, miRNA can regulate critical elements in cancer and are referred to as oncomirs. One of the best known oncomirs is the miR-17~92 cluster. This cluster is highly conserved among species and is located within chromosome 17, at 13q31.3 amplicon (discussed above) (He et. al., 2005). Over expression of the miR-17~92 cluster occurs in many tumor types, although the amount of miRNA expressed depends on the cell type and specific tumor type (Petrocca et. al., 2008; Volinia et. al., 2006; Lu et. al., 2005). miR-17~92 cluster is expressed in many different cell types, enabling the cluster to be involved in a number of functional networks. Cluster expression results in the silencing of many genes in
a number of developmental and angiogenic pathways (Petrocca et. al., 2008; Volinia et. al., 2006; Lu et. al., 2005; He et. al., 2005; Ventura et. al., 2008).

In this study, we confirmed the constitutive elevation of a number of fibrotic genes in lung tissue from patients with ILD/IPF, but not in control lung tissue or lung tissue from patients with COPD (serving as a chronic lung disease control). Thus, we predicted that miRNAs may play an important regulator of those genes.

By in silico analysis, we found that all six members of the miR-17~92 cluster target many of these fibrotic genes. To confirm this prediction, we found expression of the miR-17~92 cluster in human ILD/IPF lung tissue was reduced and that target mRNA genes were elevated, suggesting that the miR-17~92 cluster may play an important role in ILD/IPF disease.

2- Results

A- mRNA profiling of ILD/IPF patients

To understand the regulation of gene expression in ILD/IPF, we examined the mRNA expression profile between patients with ILD/IPF, COPD (chronic lung disease control) and normals (normal lung tissue resected around areas of lung cancer) and sought to determine the gene pattern and gene pathways distinguishing ILD/IPF from controls (normal and COPD tissue samples). We profiled mRNA expression from lung specimens of twenty-one lung biopsy specimens from patients with major diagnosis of UIP/IPF or non-specific interstitial pneumonia (NSIP) (which we will refer together as ILD/IPF) from the
Lung Tissue Research Consortium (LTRC). The forced vital capacity (FVC pre-bronchodilator, % predicted) was provided in the annotations of the samples and we stratified the samples according to FVC, as mortality in the disease correlates to pulmonary function (Schmidt et. al., 2010).

Pathological lung tissue specimens were segregated into three FVC categories <50%, most severe; 50-80%, moderate; and >80%, mild. For comparison, we also examined ten specimens from patients with a diagnosis of COPD to control for the effects of smoking and to control for chronic lung disease. We also obtained normal adjacent lung tissue from patients resected for cancer from the Cooperative Human Tissue Network (CHTN) as additional control samples. Quality assurance checks included examination of RNA integrity, cDNA yield after amplification, and visual inspection of the Affymetrix raw data files for outliers due to high background or other hybridization artifact.

The patients with ILD/IPF were subjected to hierarchical cluster analysis along with the profiles from COPD patients and ‘control’ patients. We found that many genes were significantly altered in ILD tissues relative to controls by 1.5 fold or greater, with a positive false discovery (pFDR) of less than 0.1. In this study, we found 1423 differentially expressed genes (DEGs) where 628 genes were repressed and 952 genes were highly expressed compared to controls (Figure 10).
Figure 10. Unsupervised hierarchical clustering with 1423 differentially-expressed genes between ILD patient and normal control lung samples. Each row represents the expression profile of a gene across 29 samples and each column represents a sample. The sample IDs and clinical information (FVC group and diagnosis) are listed below the heatmap. Based on the dendrogram, the samples can be further separated into four subgroups below the heatmap indicated by red (other IIP), green (less severe UIP/IPF [FVC 2 and 3]), blue (more severe UIP/IPF [FVC 1]) and black (normal control) bars. On the heatmap, the colors refer as, Red: low expressed genes, black: unchanged and green: highly expressed genes.
Examination of the ILD/ILD/IPF profiles appeared to cluster by forced vital capacity (severity group). To validate the stratification of gene expression in the three FVC groups, we performed a principal component analysis (PCA) of the ILD/IPF profiles (Figure 11). This procedure reduces the observed variables into a smaller number of artificial variables without loss of information (Shlens, 2005). This analysis showed that ILD/IPF patients of FVC group 1, 2, and 3 (group 1 FVC <50% \( \text{ILD-1} \) of predicted; group 2 FVC 50-80% \( \text{ILD-2} \) of predicted; group 3 FVC > 80% \( \text{ILD-3} \) of predicted) tended to cluster within each FVC group. The principal component analysis also showed that ILD/IPF lung mRNA profiles segregated from lung mRNA profiles from COPD patients. It is worth mentioning that COPD patients had low FVC and were segregated into two categories <50%, most severe; and 50-80%, moderate. Beside, most of the patients have similar smoking histories as IPF patients.
Figure 11. IPF/ILD patients with distinct forced vital capacity have different patterns of gene expression. The mRNA profiles were analyzed with a 2-way ANOVA and principal component 1 of the gene expression data was plotted as a function of principal component 2. The Bioconductor software was used for this analysis. These plots represent projections onto two dimensions of higher dimensional data. The three FVC categories were FVC 3<50%, most severe; FVC 2 50-80%, moderate; and FVC 1 >80%, mild disease.
B- Expression of many genes involved in lung fibrosis

Recent studies reveal that IPF is not an inflammatory disease, but instead it is likely a chronic wound-like injury involving lung epithelial cells and fibroblasts, where the inability of epithelial cells to repopulate and repair the injury is the basic problem (Agostini et. al., 2006).

To date, reports show elevation of certain genes in the lungs of patients with ILD/IPF, including connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) (Wynn, 2008). CTGF is a prototypic member of (CCN) protein family that been regulated by many fibrotic mediators such as thrombin and Transforming Growth Factor-beta (TGF-β) that stimulates myofibroblast differentiation and production of matrix proteins (Leask et. al., 2003). Other genes and proteins elevated in ILD/IPF lung tissue include molecules like VEGF. VEGF is an effective growth factor in epithelial cells that regulates angiogenesis stimulation and vascular permeability (Voelkel et. al., 2006). VEGF plays an important role in lung development, vascular permeability and pathological angiogenesis (Antoniou et. al., 2009; Voelkel et. al., 2006; Millauer et. al., 1993; Yancopoulos et. al., 2000). Recent studies suggest that VEGF concentrations may reflect the severity of ILD/IPF and predict its progression (Ando et. al., 2010). Interestingly, VEGF enhances CTGF expression that activates fibroblast proliferation and extracellular matrix production (Suzuma et. al., 2000; Wand et. al., 1998). Downregulation of VEGF
in an IPF mouse model by inhibiting VEGF receptors can attenuate lung fibrosis (Hamada et. al., 2005; Wang et. al., 1998; Koyama et. al., 2002).

In this study, we aimed to distinguish ILD/ILD/IPF lung mRNA profiles from lung tissue from COPD patients and normal patients. Therefore, we examined the expression of several fibrotic genes that are associated with ILD/IPF in our patient samples. These genes were known to be highly expressed in IPF, including CTGF; VEGF; Elk-3; Matrix metalloproteinases (MMPs); Interleukin-6 (IL-6); Thrombospondin-1 (Tsp-1); collagen-1, -3, -13; tuberous sclerosis complex gene (TSc-2); Fas, Interleukin-1R2 (IL1-R2); Ets family of transcription factors-1 and -2 (ATS, 2000; ATS, 2003; Wynn et. al., 2008; Kaminski et. al., 2006; Oettgen et. al., 2006; Cosgrove et. al., 2002; Moodley et. al., 2003; Liu et. al., 2009; Carsillo et. al., 2000; Garcia et. al., 2004; Cushing et. al., 2010; Sato et. al., 2001). We found that the expression of most of these fibrotic genes was increased in a dose-dependent manner proportional to disease severity denoted by FVC (Figure 12A, B and C). At the same time, we noticed that other genes like CTGF, VEGF, and Col13a1 are elevated in lung tissue from patients with moderate, but not mild, disease. Notably, their expression appeared to further increase in the most severe cases.
Figure 12. Gene expression in lung tissue from ILD/IPF patients. A, B and C include ILD/IPFILD/IPF patients divided according to FVC into group 1, 2, and 3: (group 1 FVC <50% (ILD-1) of predicted; group 2 FVC 50-80% (ILD-2) of predicted; group 3 FVC > 80% (ILD-3) of predicted) against normal control patients. Gene expression was tested using RT-PCR and normalized to GAPDH and CAP gene.

Continued
To verify that genes differentially-expressed in ILD/IPF fibrotic lung tissue led to protein production, we analyzed the protein level of selected genes by Western blot in lung tissue from patients in each of the three groups based on FVC. Because we were limited in some patient samples, samples were selected according to availability.

In correlation to mRNA levels, the levels of CTGF and Tsp-1 were elevated according to disease severity. Patients with moderate and severe ILD/IPF disease had higher protein levels of fibrotic genes (Figure 13).
Figure 13. Validation of gene expression in ILD/IPF at a protein level. Western blot analysis was performed using different antibodies to determine the expression of some fibrotic genes at a translational level. G1: group 1 FVC <50% (ILD-1) of predicted; G2: group 2 FVC 50-80% (ILD-2) of predicted; group 3 FVC > 80% (ILD-3) of predicted) against N: normal control patients.

C- Network activation in IPF lungs based on mRNA profiles

We calculated the mean gene expression from ILD/IPF profiles and divided these values by the mean expression observed in the control samples. These values were used to identify key biological pathways that are likely to be active in our ILD/IPF patients. Ingenuity software analysis scored ten pathways with acceptable P-values <0.01 (Table 4).
Table 4. Biological pathways implicated in ILD: preliminary comparison of ILD profiles relative to control profiles. The mean expression value for each gene within a sample grouping (ILD/IPF/ILD/IPF or CTRL) was fit into an analysis of variance model. Confidence intervals were calculated across all results using Tukey’s Honest Significant Differences calculation in R/Bioconductor, producing an adjusted p-value. The 10 pathways with the highest significance are shown.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>p-value±STD</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>Acute Phase Response Signaling</td>
<td>2.64E+00</td>
</tr>
<tr>
<td>Integrin Signaling</td>
<td>2.60E+00</td>
</tr>
<tr>
<td>Hepatic Fibrosis / Hepatic Stellate Cell Activation</td>
<td>2.41E+00</td>
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<tr>
<td>IL-6 Signaling</td>
<td>2.26E+00</td>
</tr>
<tr>
<td>B Cell Receptor Signaling</td>
<td>2.11E+00</td>
</tr>
<tr>
<td>Complement System</td>
<td>2.09E+00</td>
</tr>
<tr>
<td>PPARγ/RXRα Activation</td>
<td>1.96E+00</td>
</tr>
<tr>
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</tr>
<tr>
<td>cAMP-mediated Signaling</td>
<td>1.87E+00</td>
</tr>
</tbody>
</table>

The principal component analysis distinguishes ILD/IPF lung mRNA profiles from those from COPD patients. These pathways are consistent with those known to be active in the lungs of ILD/IPF patients. These pathways include myofibroblast proliferation, extracellular matrix synthesis, developmental pathways, and angiogenesis. Genes implicated in these pathways strongly suggest a large role for mesenchymal cell activation and proliferation, but do not discriminate the proposed origins of the regulation of this myofibroblast activity;
recruitment of fibroblasts/fibrocytes from the circulation, or the presence of Epithelial cell to Mesenchymal cell Transition (EMT). Importantly, many of these pathways and processes have been previously identified in profiling studies from lung tissue in patients with ILD/IPF (Selman et. al., 2006; Selman et. al., 2008; Willis et. al., 2005). Active genes such as Notch and VEGF signaling are consistent with active or aberrant developmental programs, angiogenic programs and endothelial cell targeting and turnover (Cosgrove et. al., 2004; Magro et. al., 2006). The genes responsible for triggering the ‘hepatic fibrosis/satellite cell activation’ pathway emphasize the importance of TGF-β, TGF-α, EGF, and endothelin signaling in our ILD/IPFILD/IPF samples. These signaling molecules regulate many of the effectors of extracellular matrix remodeling including type I and type III collagen, and matrix metalloproteinase-2 and -7.

D- Our Hypothesis

Since there does not seem to be a single gene aberrant in IPF responsible for the ILD, but instead, there are changes in expression of multiple genes, the elucidation of a connecting hypothesis is needed. We predicted that aberrant miRNA expression may be a central process in ILD/IPF to regulate almost global gene expression.
E- Identification of candidate miRNAs involved in the upregulation of fibrotic genes in ILD/IPFILD/IPF patients

IPF is considered to be a Multifactorial Inheritance and Genetic Disease where multiple gene loci are involved and inherited in a non-mendelian pattern (Hutyrov’a et. al., 2002; Garantziotis et. al., 2009; Olson et. al., 2007). Evolutionarily, small non-coding miRNAs are important in regulating multiple gene loci. An expression database of over 600 miRNAs has been discovered with at least 1000 miRNAs estimated as compared to over 30,000 readouts for coding mRNAs, with the understanding that these miRNA regulate at least 40% of genes.

Since we found changes in the expression of multiple genes, we speculated that transcriptional or post-transcriptional gene regulation effected the expression of these genes. Recent studies in a variety of disease areas focus on miRNAs. Since many genes are expressed in ILD/IPF lung tissue, we predicted that the expression of the certain miRNAs was decreased in lung tissue from patients with IPF. Using several miRNA target prediction algorithms software programs (Target scan, Miranda, Pictar, miRbase, miRNA map and Diana microTest) (Enright et. al., 2003; Lewis et. al., 2003; Stark et. al., 2003; john et. al., 2004; Kiriakidou et. al., 2004; Rajewsky et. al., 2004; Dongen et. al.,2008), we found that the miR-17~92 cluster targets (in red) numerous fibrotic genes that are elevated in IPF, including collagen, TGF-β, and MMPs (Table 5).
Our data-driven approach linking quantitative phenotype (%FVC) with *miRNA* profiling of lung tissue in patients with IPF identified the *miR-17~92* cluster as a novel target in pulmonary fibrosis.

**F- mRNA and miRNA profiling of IPF lungs**

The samples profiled for mRNA were also profiled for *miRNA* by a RT-PCR-based method. The hierarchical clustering results for sixteen of the ILD/IPF patient samples and five control samples are shown in Figure 14.
Figure 14. Hierarchical cluster analysis of miRNAs in lung tissue from patients with ILD or uninvolved-normal tissue from lung cancer patients (CTRL). Identical samples analyzed for mRNA expression were analyzed for miRNA expression by qRT-PCR. Correction of the raw PCR cycle threshold (CT) scores included normalization by the method of Vandesompele (Vandesompele et al., 2002). In this study, 24 miRNAs were differentially expressed among the samples as determined by Student’s t-test followed by the Benjamini & Hochberg multiple test correction. The Vandesompele normalization method was implemented using the SL qPCR Bioconductor library (Kohl, 2007). Color key: dark green, highest expression; dark red, lowest expression.

Similar to mRNA profiles, the miRNA profile from lung tissue of patients with ILD/IPF clustered to the right of the figure, while the control profiles grouped to the left, suggesting an emergent miRNA signature or profile in ILD/IPF lung samples. This analysis demonstrates the ability to capture miRNA profiles from frozen samples, stratify the data, and relate the miRNA profiles to mRNA profiles.
Hierarchical analysis of the ILD/IPF data by FVC functional group suggests emergence of specific miRNA profiles. Importantly, miR-19b, miR-20a, and miR-106b (miR-17 paralog) are highly-expressed in control lung tissue, but are markedly reduced in lung tissue from patients with ILD/IPF. These miRNA profiles implicate the miR-17~92 cluster as a potential novel target that is reduced in patients with ILD/IPF. Reduced expression of this miRNA cluster may predictably-enhance expression of gene networks targeted by these miRNAs (Table 4).

At the same time, we identified 125 differentially-expressed miRNAs (DEmiRNAs) where 82 miRNAs were highly expressed in ILD patient samples compared to the controls (Figure 15). We noticed a significant clustering in miRNA expressing between COP and ILD profiles. Interestingly, the clustering results from DEmiRNAs failed to distinguish the disease severity groups (FVC groups) as DEGs.
Figure 15. Unsupervised hierarchical clustering result of 125 differentially expressed miRNAs. The sample IDs and clinical information (FVC group and diagnosis) are listed below the heatmap. Red or green colors indicate either higher or lower expression levels of the miRNA. Samples are well separated into control and ILD patient groups.

Because *miR-106b* and *miR-20a* are highly expressed in control samples and reduced in ILD/IPF samples (predicting higher target mRNA), we explored the
affected predicted pathways (Figure 16) using DAVID bioinformatics resources (Huang et. al., 2009). Here the cytokine-cytokine receptor interaction pathway is predicted as mRNA targets for each of these miRNAs. Such informatics analysis demonstrates that a given miRNA can predictably interact with many mRNAs and thus impact many biological pathways. This information may provide indirect clues to stage specific markers that will be useful for diagnosis or prognosis. Nonetheless miRNA profiles may provide clues to key pathologic processes and as we interpret miRNA in the context of mRNA profiles, we can link regulatory pathways that may regulate network activation in patient samples.

Figure 16. Biological pathways associated with miR-106b and miR-20a. Pathways are returned on the basis of mRNA similarity and are listed based on the number of potentially interacting mRNAs known to be part of the given pathway.
G-Validation of expression of miR-17~92 cluster by quantitative RT-PCR, in situ hybridization and Immunohistochemistry (IHC)

To validate the down-regulation of the six miRs of the miR-17~92 cluster in ILD/IPFILD/IPF patient lung tissue, quantitative real time PCR analysis was carried out with miRNA specific primer/probe sets under pre-optimized conditions. Primer/probe sets were pre-selected to exclude precursor miRNA amplification (ABI, Applied Biosystem). Same samples of ILD/IPF patient were used for this analysis. As determined by qRT-PCR, significant decreases of individual mature miRNAs of the cluster were observed (Figure 17). This data confirmed the results of the hierarchical cluster analysis of miRNAs in patient's lung tissue. As an internal reference correlative control, the expression analysis of RNU-48 and U6-snRNA were used since no change in expression was shown between ILD/IPF and normal tissue by qRT-PCR.
Figure 17. Validation of expression of miR-17~92 cluster in ILD/IPF tissue. To confirm the observed decrease in miR-17~92 cluster expression from profiling data, qRT-PCR was used. Shown is the average relative expression normalized to RNU48 internal control ±S.E.M (0.003) (Control n=11, >80% FVC n=8, 50-80% FVC n=8, and <50% FVC n=9).
To confirm the observed decrease in miRNA expression, we selected two miRNAs to investigate their expression by in situ hybridization. We selected miR-19b and miR-20a since they targeted seven and three of ten genes found in Table 4, respectively. Both of these miRNAs are encoded in the middle of the cluster. Furthermore, they are predicted to target three of the same genes. As shown in Figure 18, we observed prominent staining for both miRNAs in control tissue, and noted reduced expression in tissues samples from IPF patients with mild disease (>80% FVC). Expression of miR-19b and miR-20a was nearly undetectable in lung tissue from patients with moderate (50-80% FVC) and severe disease (<50% FVC). As expected, we failed to detect non-specific staining of miRNAs in tissue stained with scrambled probes that served as a negative control. As a positive control for the in situ hybridization, we utilized a probe recognizing miR-let-7-c. As expected, staining for this probe was similar among the IPF patient samples and control lung tissue samples.
Figure 18. *In situ* hybridization on paraffin sections of human lung tissue of ILD/IPF patients and normals. Using labeled LNA probe for let7-c (negative control, scrambled (positive control miR-19b and miR-20a) probe at X100 magnification to detect there expression in different pathological groups of ILD/IPF against normal lung tissue.

To confirm our results of mRNA:miRNA interactions in ILD/IPF patients samples, we next determined putative targets (VEGF) of miR-19b by colocalization analysis with immunohistochemistry. Knowing that miR-19b is reduced in lung tissue from ILD/IPF patients and increased in normal lung tissue, and vice versa for VEGF, we analyzed serial sections with immunohistochemistry.
using antibodies directed against VEGF. There was reduced miR-19b expression in severe ILD/IPF than normal lung tissue. We then performed co-localization experiments with miR-19b and VEGF (Gerad et. al., 2008 and 2009) and found that the cells in ILD/IPF samples making VEGF did not contain miR-19b and vice versa (Figure 19). These data suggest that miR-19b targets VEGF and can repress its expression.

![Figure 19. Co-localization of miR-19b and VEGF in different groups of ILD/IPF lung tissue against normal lung tissue. Immunohistochemistry was applied using VEGF antibody followed by in situ hybridization of miR-19b on the same paraffin section to test the co-localization of miR-19b and VEGF in ILD/IPF disease. Brown staining indicated positive staining for VEGF, collagen fibers stained blue and miR-19b stained brown/purple dots.]
3. Discussion

IPF is an aggressive interstitial lung disease that leads to inexorable progression and death. It is distinguished by its progressive scarring of the lungs, without identifiable cause (Selman et. al., 2008). However, the etiology and pathogenesis of IPF is poorly understood. In fact, the median survival is <3 years from diagnosis (Kuwano et. al., 1996). While there are many hypotheses as to the etiology of the disease, experts speculate that IPF is a disease of wound-like injury of the lung with primary failure to resolve the injury by epithelial cell repair mechanisms (Kaminski et. al., 2000; Magro et. al., 2006; Maher et. al., 2007; Kaminski et. al., 2000; Magro et. al., 2006; Maher et. al., 2007). In the absence of repair, myofibroblasts are recruited to cover the wound with collagen. It is likely that multiple factors are involved in the development of IPF including epithelial-mesenchymal transition (EMT) (Willis et. al., 2007), fibroblast proliferation and activation (Ramos et. al., 2001), and failure of wound repair and re-epithelialization (Bringardner et. al., 2008).

While the pathogenesis of the disease is not known, there are some emerging clues that point to cellular senescence as a contributor to dysfunctional lung repair. Recent work clarifies a role for telomeres in small numbers of familial IPF patients, which gives insight into the importance of cellular robustness in lung repair and recovery from the disease. In addition, investigators find that mutations in surfactant protein-C leads to lung fibrosis underscore the importance of lung epithelial cells in the disease. Given that lung epithelial cell
repair is important in IPF, we focused on defining the molecular regulation of epithelial cell development and regeneration. Others found that the miR-17~92 cluster plays an important role in the developmental production of lung epithelial cells (Ventura et. al., 2009; Lu et. al., 2007; Selman et. al., 2001), while here we report that the miRNA cluster was decreased in lung tissue from IPF patients. Many studies have found changes in the expression of numerous genes in tissue with IPF. Many of the increased genes are associated with wound repair, including MMPs, collagen, and growth factors among others (Henry et. al., 2002; Voeghtly et. al., 2009). Others focus on genetic changes in the peripheral blood that could be used as diagnostic and/or prognostic factors (Rosa and Kaminiski 2006). Since a single miRNA can bind to negatively-regulate 10-100 genes, we focused on these molecules as regulatory factors that impact several genes.

We first compiled a list of highly-expressed genes in IPF and subjected them to prediction database analyses to determine predicted miRNA targets. We found that miRNAs in the miR-17~92 cluster are predicted to target many of the highly-elevated genes associated with ILD/IPF. It is also noteworthy that mice lacking the miR-17~92 cluster lack normal lung epithelial cells and have hypoplasia of the lung (Ventura et. al., 2008). In contrast, overexpression of the cluster results in a hyper-proliferation of lung epithelial cells (Lu et. al., 2007; Carraro et. al., 2009). The predicted role of the miR-17~92 cluster is critical in lung epithelial cell homeostasis and development.

The miR-17~92 cluster regulates tumor angiogenesis (Virginie et. al., 2010). As opposed to the majority of miRNAs that are decreased in tumor environments,
miR-17~92 clusters is increased (Mendel et. al., 2005). It has been reported to target CTGF, VEGF and TGFβ expression which is highly expressed in lung tissue from patients with ILD/IPF (Hua et. al., 2006; Kaminski et. al., 2006; Selman et. al., 2008).

Given our prediction database search and previous observations, we hypothesized that expression of the miR-17~92 cluster was decreased in lung tissue of patients with ILD/IPF. As expected, we found a significant decrease of each individual miRNA contained in the cluster. In this study, we compared the differential expression of the miR-17~92 cluster in different stages of fibrosis in human IPF. We used different high throughput Hierarchical cluster analysis, qRT-PCR and in situ hybridization of miRNAs to classify and define miR-17~92 cluster profiling in ILD/IPF.

Indeed, we found the repression of miR-17~92 cluster expression was in reverse proportion with targeted gene expression. Despite these results, further, in-depth analysis is needed to understand how these genes are being regulated in ILD/IPF through the cluster, what causes miR-17~92 cluster to be down-regulated in ILD/IPF, and how that is related to increase severity of the disease.

The remaining part of my dissertation will focus in detail on the dysregulation of the cluster in ILD/IPF, revealing some unknown mechanism on how the cluster is being regulated in ILD/IPF, and at the end suggesting clinical therapeutic strategies that can be used to treat ILD/IPF patients.
4. Conclusion

In this chapter, we reveal the expression of the *miR-17~92* cluster in ILD/IPF patient lung tissue. We found that the cluster is down-regulated according to severity of IPF disease. At the same time, our data support others in showing the high expression of many fibrotic genes (such as *CTGF*, *VEGF*, Collagens) and for the first time, we found that this increase in gene expression positively correlated to disease severity and may play an important role in IPF progression.

These results encourage us to study the regulation of the *miR-17~92* cluster and its how it may target predicted fibrotic genes. Manipulation of the cluster in an IPF cell line would be the ideal steps to prove our hypothesis.
CHAPTER FOUR: THE RELATIONSHIP BETWEEN THE *miR-17~92* CLUSTER AND THE UPREGULATION OF FIBROTIC GENES IN IPF HUMAN LUNG FIBROBLASTS

1. Introduction

Recent studies of *miRNA*s show the importance of their expression on the silencing of genes involved in the manipulation of many vital cellular pathways. Altered expression of *miRNA*s has revealed the mechanisms of numerous cellular and developmental processes such as organ morphogenesis and signaling pathways (Stefani et. al., 2008) and is related to many pathological diseases such as leukemia, diabetes and cardiovascular diseases (Croce et. al., 2009., Condorelli et. al., 2009; Thum et. al., 2008).

In lung fibrosis, the role of *miRNA*s is not clear. Thus, understanding and characterizing the role of specific *miRNA*s in lung fibrosis will be considered an essential addition in clarifying the complexity of lung fibrosis pathogenesis and diagnosis.

We explored the role of the *miR-17~92* cluster in IPF lung samples and we found that it is repressed, while its target genes were upregulated with increasing severity of IPF disease. These results suggest that the *miR-17~92* cluster is a
central mediator in IPF pathogenesis and could be a potential target for a therapeutic treatment. To investigate this hypothesis, we manipulated the expression of the miR-17~92 cluster in human IPF lung fibroblasts. Interestingly, we found that this manipulation had a significant effect on IPF lung fibroblasts at both the morphological and molecular levels.

2. Results

A-The use of human lung fibroblast cell lines as an IPF model

In our previous study, we found that the expression of the miR-17~92 cluster was dramatically repressed in IPF lung tissue compared to normal lung tissue, while miR-17~92 predicted target genes were highly upregulated in the same samples. This led us to believe that the miR-17~92 cluster could be the cause of increased expression of many fibrotic genes in IPF.

Since collagen deposition in IPF is due to enhanced fibroblast activity leading to extracellular matrix deposition (Murray, 2008), we analyzed the expression profile of the miR-17~92 cluster in normal lung fibroblasts (ATCC# CCL-201 and CCL-204) and those derived from patients with IPF (ATCC# CCL-134 and CCL-191). Similar to the expression in lung tissue from patients with ILD/IPF, there was a dramatic decrease in the expression of each miRNA from the miR-17~92 cluster in the IPF fibroblast cells (CCL-134) compared to normal lung fibroblasts (CCL-204). A similar decrease was observed in the IPF fibroblast CCL-191 cell line compared to the normal lung fibroblast CCL-201 cell line (Figure 20).
Notably, the CCL-204 normal fibroblasts and the CCL-134 IPF fibroblasts were derived from patients that were 35 and 26 years of age, respectively, while the normal fibroblasts (CCL-201) and IPF fibroblasts (CCL-191) cell lines were from older individuals (both 48 years of age). Both cell lines revealed the same result pattern, but IPF fibroblasts (CCL-191) and normal fibroblasts (CCL-201) showed high levels of senescence and high sensitivity to transfection reagents and were problematic to maintain in tissue culture for long periods of time. We think this is because of the old ages of the patients from which they were derived. Thus, we chose to use CCL-204 and CCL-134 cell lines for subsequent experiments.
Figure 20 (A and B). The expression of the miR-17-92 cluster in human lung fibroblasts (Normal and IPF). qRT-PCR was used to measure the expression of the cluster in human lung fibroblast cell lines. Shown is the average relative expression number (RCN) normalized to RNU48 internal control. N=3
Next, we tested the translational level of some fibrotic genes of interest using Western blot analysis. Not surprisingly, we found the amount of those genes were higher in the IPF fibroblast cell line than the normal fibroblasts (Figure 21).

**Figure 21.** Gene expression in human lung fibroblasts at the translational level. Western blot analysis was performed using specific antibodies to determine the expression of various fibrotic genes at the level of translation in human lung fibroblasts. β-actin used as the loading control. N=3
B-Introduction of the *miR-17~92* cluster in IPF-derived lung fibroblasts results in phenotypic changes in the cells

Morphology is a critical feature of cellular phenotype and can reflect many oncogenic transformations (Logan et. al., 1994). It is worth mentioning that the morphology of normal fibroblasts and IPF fibroblasts was markedly different. Normal lung fibroblasts had a normal, elongated bipolar spindle shape, while the IPF lung fibroblasts had a flat shape with many pseudopodia and filopedia extensions (Figure 22).

Upon over-expression of the cluster, we noticed changes in cell morphology in the IPF cells. Notably, untransfected IPF lung fibroblasts had numerous filopedia compared to normal lung fibroblasts. Introduction of the *miR-17~92* cluster in the IPF cell line resulted in loss of filopedia and a transformation back to the spindle shape, similar to normal morphology. Interestingly, normal lung fibroblasts transfected with the cluster did not have an altered phenotypic appearance (Figure 23). Green fluorescent protein (GFP) transfection was done side-by-side to test the transfection efficiency.
Figure 22. Morphology of lung fibroblasts. A normal lung fibroblast has the standard elongated shape with pointed ends while an IPF lung fibroblast has a flat, wide shape with many pseudopodia and filopedia extensions. Arrows in red refer to filopedia formation in IPF fibroblasts.
Figure 23. Effect of the miR-17~92 cluster on the morphology of lung fibroblasts. Transient transfection of the miR-17~92 cluster into lung fibroblasts changes the morphology of IPF fibroblasts only.

Thus, IPF lung fibroblasts expressing the miR-17~92 cluster assumed a more normal morphology, possibly due to reduced expression of fibrotic genes.

C-Effect of the miR-17~92 cluster on IPF-derived lung fibroblasts at the molecular level

Next, we tested the effect of the miR-17~92 cluster on predicted target genes and examined if the cluster does indeed targets those genes and affect their expression. We treated fibroblasts with increasing concentrations (0.5 μg, 1.0 μg, 1.5 μg) of the miR-17~92 cluster for 24 and 48 hours. We found that the optimal
conditions with the highest transfection efficiency was 0.5 μg of miR-17~92 cluster for 48 hours, and thus we used this condition in all subsequent experiments.

After transfection, total RNA was isolated to test by RT-PCR the expression of miR-17~92 and other well-known genes. Also, protein lysate was collected from these cells to test the translational level of those genes and identify the regulation level of the miR-17~92 cluster on those genes in IPF. We found that expression of the miR-17~92 cluster in IPF fibroblasts significantly reduced the mRNA expression of VEGF, CTGF, Col1a1, Col13a1, TSP-1, ELK-3, TERT, and ETS-1 compared to control untransfected cells, mock-transfected cells, and cells transfected with empty vectors. At the same time, the expression of these genes was not elevated in normal lung fibroblasts and remained relatively unchanged after transfection with the miR-17~92 cluster. As a negative control, we examined expression of HIF-1α, which is not a predicted target of the miR-17~92 cluster. As predicted, there was no significant change in HIF-1α expression following transfection with the miR-17~92 cluster in either normal or IPF lung fibroblasts (Figure 24).
Figure 24 (A-J). Real-Time PCR results of fibrotic genes after re-introduction of miR-17~92 cluster into human lung fibroblasts at 0.5µg for 48 hrs. Fibroblasts were transfected as: mock (transfection reagent without DNA vectors), Vector (transfection with 0.5 µg of pcDNA 3.1 vector only without miR-17~92 cluster) and miR-17~92 cluster (transfection with 0.5 µg pcDNA3.1 vector contained miR-17~92 cluster). Using SAbiosciences RT-PCR primers normalized against CAP and GAPDH internal control. N=3, * P< 0.0014.
Figure 24. Continued

C.

D.

Continued
Figure 24. Continued

E.

Tsp-1

F.

ELK-3

Continued
Figure 24. Continued

G.

H.

Continued
Figure 24. Continued

I.

![ETS-2 graph](image)

J.

![HIF-1α graph](image)
At the same time, we tested the translational level of those genes by Western blot after introducing the miR-17~92 cluster back to the cell. A dramatic decrease of the protein was observed after adding the cluster back into the IPF fibroblasts (Figure 25).

Figure 25. Gene expression in IPF lung fibroblasts at the translational level. Western blot analysis was performed using specific antibodies to determine the expression of fibrotic genes at the level of translation in IPF lung fibroblasts. β-actin was used as the loading control. n=3
D- Restoration of the *miR-17~92* cluster back into IPF fibroblasts reduces senescence-associated β-Galactosidase staining and increases proliferation

Besides the morphological and molecular changes after transfecting these cells with the *miR-17~92* cluster, we noticed increases in proliferation in both normal and IPF fibroblasts. Many studies have shown that senescence is a risk factor of developing IPF (Minagawa et. al., 2010). Thus, we examined the modulation effect of the *miR-17~92* cluster on SA-β-gal activity, a characteristic feature of senescence growth arrest. We found that overexpression of the *miR-17~92* cluster decreased the percentage of SA-β-gal–positive cells by 15% in normal fibroblasts and by 50% in IPF fibroblasts compared to controls. At the same time, we noticed an increase in proliferation rate after transfection with the *miR-17~92* cluster in both fibroblast cell lines using the MTT and scratch assays. This supports other studies showing the significant role of the *miR-17~92* cluster in cell cycle regulation and angiogenesis (He et. al., 2005) (Figure 26).
Figure 26. miR-17–92 cluster modulates senescence and proliferation rate in human lung fibroblasts. A. SA-β-gal staining after miR-17–92 cluster transfection into IPF lung fibroblasts. B. Statistical analysis for SA-β-gal staining. C. Scratch assay was applied to test the proliferation rate of IPF lung fibroblasts after miR-17–92 cluster transfection. D. MTT assay on lung fibroblasts after transfection of the cluster. n=3, *P< 0.003
Figure 26. Continued

C.

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D.

![Graph showing absorbance at 570 nm for different conditions with legend: Red square for Normal lung fibroblast CCL-204, Black square for IFF lung fibroblast CCL-131]
3. Discussion

In our previous data we showed that the miR-17~92 cluster was significantly repressed in human IPF tissue samples. This repression was inversely related with an increase in severity of the disease. Thus, it shows that the miR-17~92 cluster may have a dramatic role in diagnosis and progression in IPF. Manipulating the cluster in vitro in the IPF lung cell line model was crucial to verify our hypothesis and unravel the role of specific involvement of the cluster in IPF pathogenesis.

We observed a decrease in the expression of the miR-17~92 cluster in a lung fibroblasts derived from patients with IPF compared to normal lung fibroblasts. This observation was essential to proceed in our study and to consider it a good model for IPF disease in vitro. Through manipulation of miRNA cluster expression, we further demonstrated a reciprocal relationship between the expression of predicted target genes and the miRNAs contained in the cluster. Not only did we detect molecular changes in the transfected fibroblasts, but also we observed phenotypic changes in the IPF fibroblasts that were transfected with the cluster, resulting in a normal appearing cellular phenotype. In addition, we were able to alter the senescence and the proliferation rate of lung fibroblasts by re-introducing the miR-17~92 cluster, which supports other studies focused on the role of the miR-17~92 cluster in cell cycle regulation and angiogenesis (He et. al., 2005). Therefore, dysregulation and repression of the cluster expression in ILD/IPF lung tissue and fibroblasts needs to be explored in more detailed, in
order to understand the factors that control the expression of the cluster and cause it to be repressed in IPF.

4. Conclusion

This *in vitro* study to characterize a model of ILD/IPF was essential to proceed in our project. It served as a good model to manipulate and alter the cluster at optimal concentration and duration in the fibroblasts, beside, it provided a proof of concept and may suggest a novel therapeutic treatment for IPF.

Our data suggest that *miR-17~92* cluster is down regulated in IPF lung fibroblasts compare to normal fibroblasts, which is similar to what we found in human ILD/IPF tissue samples. In consequence, the next question was what causes this repression, especially when the only known regulator (c-myc) is being expressed and phosphorylated/active in both cell lines and in IPF lung fibroblast and controls. According to our data, we suggest a novel model involving the *miR-17~92* cluster expression as an underlying mechanism in ILD/IPF in the next chapters.
CHAPTER FIVE: EPIGENETIC REGULATION OF $\textit{miR-17-92}$ CLUSTER IN IDIOPATHIC PULMONARY FIBROSIS (IPF)

1-Introduction

Epigenetic regulation describes stable alteration in gene expression during cell proliferation, development and cellular phenotype (Wolffe et. al., 1999). These changes are transmitted through multiple generations by many mechanisms beyond DNA sequence where the same genome gives rise to stable cell types (LI et. al., 2002). Epigenetic regulation of gene transcription is also a way that the organism expresses impact from its environment and behavior on its genome. The three basic mechanisms of epigenetic regulation is by methylation of CpG islands in gene promoter regions (Bird et. al., 2002), histone acetylation rendering genes inaccessible to transcriptional machinery (Lund et. al., 2008), and regulation by small non-coding RNAs like $\textit{miRNA}$ (Langevin et. al., 2010; Han et. al., 2007), which either degrade targeted mRNAs or block their translation to proteins.

In somatic cells, most genes are fixed transcriptionally. However, genes, as cell cycle checkpoint genes, are regulated by exogenous growth factors that provide dynamic cell responses to these external stimuli (Bird et. al., 2002; Lund
et. al., 2008). As mentioned, there are many epigenetic switches involved in this regulation. We focused on DNA methylation since it is an area of opportunity to understand gene regulation in the lungs of patients with ILD/IPF.

DNA methylation occurs at carbon-5 of cytosine residues found in CpG islands. This step is facilitated by DNA methyltransferases (DNMTs) (Yoder et. al., 1997; Bird et al. 1993). Many studies relate DNA methylation to diseases such as oncogenesis, aging, fibrosis and embryogenesis (Jones et al., 2002; Bachman et. al., 2001).

DNMTs bind preferentially to damaged or mismatched DNA sequences and inhibit DNA promoter region access through methylation (Jams et. al., 2003; Vélicescu et. al., 2002). In addition to regulating gene expression, DNMTs can inhibit miRNA expression. Epigenetically, silenced miRNAs are markers of disease (Han et. al., 2007). For example, in squamous cell carcinoma, miR-137 promoter is methylated and considered a molecular defect that contributes to disease progression (Langevin et. al., 2010). In previous chapters, we showed a dramatic reduction of the miR-17~92 cluster in human ILD/IPF tissue and fibroblast cell lines compared to normal, suggesting an epigenetic process. Indeed, we found remarkable increases in CpG island methylation of the miR-17~92 promoter in ILD/IPF patients and IPF lung fibroblasts (Figures 27a and b).

In many epigenetic studies, inhibitors of DNA methylation are used to reactivate silenced genes. DNA methylation inhibitors are used therapeutically and include 5’-azacytidine and its analog 5’-aza-2’-deoxycytidine (decitabine) (Sorm et. al., 1964). These drugs are used as chemotoxic agents
(Constantinides et. al., 1977; Jones and Taylor, 1980). Soon after the discovery of their ability to inhibit $DNMT$s activity and inhibit methylation events in the promoter of target genes, they have been approved for clinical use in the treatment of many diseases, including cancer (Muller et. al., 2006; Oki et. al., 2007).

2. Results

A-Altered DNA methylation pattern of the CpG Islands of the $miR-17\sim92$ cluster promoter in lung tissue from patients with ILD/IPF

To address potential mechanisms accounting for reduced expression of the $miR-17\sim92$ cluster in IPF fibroblasts, we first targeted c-Myc expression, as previous studies found that c-Myc transcriptionally regulates the expression of the $miR-17\sim92$ cluster (O'Donnell et. al., 2005; Mendell, 2008; Dews et. al., 2006). We found that c-Myc was expressed similarly in tissue from ILD/IPF and control normal lung, as well as the IPF and normal lung fibroblast cell lines (data not shown). Thus we predicted that additional mechanisms regulated cluster expression.

We next hypothesized that epigenetic changes accounted for changed gene expression in lung tissue in patients with ILD/IPF and therefore examined of the upstream promoter region for CpG islands using two software packages: http://www.epidesigner.com/start3.html and http://genome.ucsc.edu/index.html?org=Mouse&db=mm9&hgsid=173350477 (Figure 27).
Figure 27. Analysis of miR-17~92 cluster promoter using two online applications. >80% of CpG Islands were found in promoter regions of the miR-17~92 cluster.
Interestingly, we found that more than 80% of the ~2 Kb promoter regions were heavily occupied with CpG islands. Thus, we hypothesized that the CpG island in the promoter region of the miR-17~92 cluster may be aberrantly methylated in lung tissue from patients with ILD/IPF to account for the miR-17~92 cluster down-regulation.

As expected, there was increased methylation of the CpG islands for the miR-17~92 cluster in the two ILD/IPF patient groups with the lowest FVC measurements (50-80% FVC and <50% FVC). Surprisingly, the majority of the CpG islands of the cluster were unmethylated in ILD/IPF patients with mild disease (>80% FVC). In contrast, we found an equal distribution of unmethylated and methylated DNA in the promoter of the miR-17~92 cluster in control normal tissue, which was largely isolated from adjacent lung tissue around an area of resection for lung cancer (Figure 28).
Figure 28. Increased DNA methylation of the miR-17~92 miRNA cluster promoter in ILD/IPF lung tissue samples. DNA was isolated from control lung tissue (n=3) and individuals with ILD/IPF. The ILD/IPF samples were grouped according to disease (n=3 per group). Using Methyl-Profiler™ DNA Methylation qPCR Primer assay, the average percent of unmethylated DNA to methylated for the miR-17~92 was determined.

Since many of the tissues used as normal were pathologically normal adjacent tissue from tumor biopsy patients, this may indicate that the adjacent area of the tumor is associated with epigenetic changes. We found similar changes in methylation pattern in lung fibroblasts derived from patients with IPF compared to normal lung fibroblasts, where the miR-17~92 CpG islands were unmethylated in normal lung fibroblasts and intermediately-methylated in IPF lung fibroblasts (Figure 29).
Figure 29. Increased DNA methylation of the miR-17-92 miRNA cluster promoter in IPF lung fibroblasts. DNA was isolated from lung normal and IPF fibroblast cell lines. Using Methyl-Profiler™ DNA Methylation qPCR Primer assay, the % DNA methylation of the cluster CpG Island was detected. HM: hypermethylated, UN: UN methylated, IM: intermediate methylated. N=3. *P <0.003

Thus, our data suggested that epigenetic silencing of miR-17-92 cluster expression was from DNA methylation of the CpG islands in its promoter.

B-Inhibition of DNA methylation in lung IPF fibroblasts leads to both phenotypic and genetic changes

Since there was increased DNA methylation of the miR-17-92 cluster in ILD/IPF lung tissue samples, we next examined whether the demethylating agent 5'-deoxy-aza-cytosine (5'-deoxy-aza-CdR) could restore cluster expression and
alter the growth characteristics of IPF fibroblasts. Therefore, we treated the lung IPF fibroblast cell line with 0.5 - 2.0 μM 5’-aza-2’-deoxycytidine for 24 - 48 hours. We found the optimal drug condition was 0.5 μM for 24 hours and this condition was used for the rest of the in vitro experiment.

Importantly, inhibiting DNA methylation in IPF fibroblasts increased the expression of several miRNAs (miR-17, miR-18a, miR-19b, and miR-20a) found in the miR-17~92 cluster (Figure 30a and b). Notably, we failed to detect changes in expression of miR-19a and miR-92 using 5’-aza-2’-deoxycytidine in IPF fibroblasts, suggesting that other mechanisms are also involved in regulating expression of the miRNAs.
A. Figure 30. The expression of miR-17~92 is increased in human lung fibroblasts treated with 5'-aza-2'-deoxycytidine. RNA was collected after 5'-aza-2'-deoxycytidine treatment and miRNA expression was examined by qRT-PCR. A. Normal lung fibroblasts treated with 5'-aza-2'-deoxycytidine. B. IPF lung fibroblasts treated with 5'-aza-2'-deoxycytidine. Shown is normalized expression ± SD(0.002), N=3. *P<0.003.
To validate these results we also tested the methylation pattern of miR-17~92 cluster CpG islands after 5’-aza-2’-deoxycytidine treatment in human IPF lung fibroblasts. We found that 5’-aza-2’-deoxycytidine decreased methylation events by 60%± SD (0.0025), *P<0.003 (Figure 31).

![Figure 31. Decreased DNA methylation of the miR-17~92 promoter in IPF fibroblasts treated with 0.05 µM 5’-aza-2’-deoxycytidine. DNA was isolated from IPF fibroblasts after 24 hours treatment. Using Methyl-Profiler™ DNA Methylation qPCR Primer assay, the % DNA methylation of the cluster CpG island was detected. N=3 , ± SD *P <0.003 HM: hypermethylated, UN: UN methylated, IM: intermediate methylated.]

Since we predicted an inverse relationship between the miR-17~92 miRNA cluster and its target gene expression, we examined the expression of its predicted targets. As expected treatment with 5’-aza-2’-deoxycytidine to reduce the expression of the target genes Col1a1, Col3a1, CTGF and VEGF decreased in IPF fibroblasts. On the other hand, we failed to detect any effect of 5’-aza-2’-deoxycytidine on the expression of a different predicted target, TGF-β or Col13a (Figure 32). This may suggest other regulatory mechanism than DNA methylation.
in the regulation of TGF-β and Col13a expression. After analyzing the promoter of TGF-β and Col13a, we could not detect any CpG islands in the 5'UTR or within the promoter region. This suggests that methylation regulation does not affect TGF-β and Col13a expression.

**A.**

![Graph showing gene expression](image)

**B.**

![Graph showing gene expression](image)

**Figure 32 (A-E). Gene expression in fibroblasts derived from IPF patients.** RNA was collected from IPF fibroblast after 5'-aza treatment for 24 hours. Gene expression was tested using RT-PCR and normalized to GAPDH and CAP gene. N=3

Continued
Figure 32. Continued

C. CTGF

D. VEGF

Continued
Interestingly, we also observed phenotypic changes in the 5'-aza-2'-deoxycytidine-(5'AZA) treated IPF fibroblasts (Figure 33). Similar to IPF fibroblasts transfected with the miR-17~92 cluster, we found a reduction in filopodia in the 5'-aza-2'-deoxycytidine-treated IPF fibroblasts. Thus, altering DNA methylation pattern with demethylating agents may be a potential therapeutic option for patients with ILD/IPF.
Figure 33. 5’-aza-2’-deoxycytidine (5’Aza) treatment causes changes in the phenotype of lung IPF fibroblasts. 24hr incubation with 0.5uM 5’Aza treatment was strong enough to alter the external phenotypic morphology of IPF fibroblast to look similar to normal lung fibroblast.

C-Increased expression of DNA methyl transferases (DNMTs) may be responsible for epigenetic changes in IPF

Since the miR-17-92 cluster in IPF appeared to be methylated, we began to elucidate the underlying mechanisms for this event. As mentioned, DNA methyltransferases (DNMTs) are important in DNA methylation (Bestor et. al., 1994; Robertson et. al., 1999). In humans, the enzymatic methylation machinery is composed of three catalytically-active DNA methyltransferases, DNMT-1, DNMT-3a and DNMT-3b. Using miRNA prediction databases (Target scan, Miranda, Pictar, miRbase, miRNA map and Diana microTest), we found that these DNMTs are potential targets of the miRNAs found in the miR-17-92 cluster. We next examined DNMTs expression in normal and ILD/IPF lung tissue. Importantly, expression of DNMT-1 mRNA increased with severity of ILD/IPF disease (Figure 34). Notably, expression of DNMT-3a and DNMT-3b were
significantly elevated only in ILD/IPF patients with the most severe disease (FVC<50%).

![Graph showing DNMT expression in ILD/IPF patient lung tissue](image)

**Figure 34. DNMT expression in ILD/IPF patient lung tissue.** DNMTs gene expression was tested in ILD/IPF patients using RT-PCR and normalized to GAPDH and CAP genes. (Group 1 FVC <50% (severe) of predicted; group 2 FVC 50-80% (moderate) of predicted; group 3 FVC > 80% (mild)) of predicted). N=3. *P< 0.003

Similarly, we observed that DNMT expression was elevated in IPF lung fibroblasts compared to normal fibroblasts (Figure 35). Thus, increased DNMTs expression may contribute to CpG island methylation in the miR-17~92 cluster in ILD/IPF lung tissue.
Figure 35 (A-C). The expression of *DNMTs* in human lung fibroblasts (Normal and IPF). qRT-PCR was used to measure the expression of the DNMTs in human lung fibroblast cell lines, which were influenced by treatment with 5'-aza-2'-deoxycytidine (5'AZA). Shown is the average relative expression number (RCN) normalized to CAP internal control. Gray resemble the normal lung fibroblast and black resemble the IPF lung fibroblast. *P <0.004. \[Continued\]
Figure 35. Continued

C.

D-Expression of the *miR-17~92* cluster alters DNMT1 expression

To determine which *DNMT* were regulated by the *miR-17~92* cluster we transfected the *miR-17~92* cluster into the IPF fibroblasts and examined *DNMTs* expression. Compared to untransfected cells, mock transfected and cells transfected with empty vector, introducing the *miR-17~92* cluster into IPF fibroblasts significantly decreased the expression of *DNMT-1* (Figure 36). In contrast, we did not observe any changes in expression of *DNMT-3a* or *DNMT-3b* compared to control samples.
Figure 36. Effect of miR-17~92 cluster transfection on the expression of DNMTs in human lung fibroblasts (Normal and IPF). qRT-PCR was used to measure the expression of the DNMTs in human lung IPF fibroblasts after re-introducing the cluster (0.5 µg). Shown is the average relative expression number (RCN) normalized to CAP internal control. N=3. *P <0.004.

E- DNMT-1 expression is directly regulated by the miR-17~92 cluster

Since DNMT-1 expression appeared to be regulated by the miRNAs contained in the miR-17~92 cluster, we examined the 3’UTR of the DNMT-1 for potential seed sequences for these miRNAs. Using the target prediction algorithms of Target scan, we found that the 3’UTR of DNMT-1 contained predicted binding sites for miR-17, miR-19b, miR-20a and miR-92a, at this location of the DNMT-1 3’UTR (ENST00000359526). The 3’UTR of DNMT-1 (generously provided by Dr. Muller Fabbri, The Ohio State University) was cloned into the pGL-3 Luciferase reporter vector. Cells were transfected with either the empty vector or the vector containing a WT DNMT-1 or mutated DNMT-1 seed sequence (predicted not to bind the miRNA sequence) and co-transfected with
expression vectors containing each of the miRNAs. We observed a reduction of
~70\(^\pm\)SD (0.0021), p<0.001 of luciferase activity by \(\text{miR-19b}\) and \(\text{miR-20a}\),
~50\% by \(\text{miR-92}\) and ~22\% by \(\text{miR-17}\) compared to cells transfected with vector
only (pGL-3) (Figure 37). As a control, we failed to detect changes in luciferase
production in the presence of \(\text{miR-19a}\), and \(\text{miR-18a}\).

Indeed only \(\text{miR-17}\), \(\text{miR-19b}\), \(\text{miR-20a}\) and \(\text{miR-92a}\) regulated the \(\text{DNMT-1}\)
3'UTR reporter, whereas the rest miRNAs were non-functional. As a control,
luciferase activity was maximized when cells were transfected with the miRNAs
in combination with a construct that has a mutated target seed sequence for
\(\text{DNMT-1}\). We were next interested to test the synergistic effect of the three
miRNAs on \(\text{DNMT-1}\) since they are grouped and transcribed in one cluster and
there seed sequence complement and target the same sequence of \(\text{DNMT-1}\)
3'UTR. Amazingly, there was a notably strong synergistic inhibition of luciferase
activity by \(\text{miR-17}\), \(\text{miR-19b}\), \(\text{miR-20a}\) and \(\text{miR-92a}\) combined together
(Figure 37).
Figure 37. *miR-17, miR-19b, miR-20a and miR-92a regulate DNMT-1 expression*. The wild-type (WT) or mutated (mut) 3’UTR for DNMT-1 was cloned into the pGL-3 Firefly luciferase vector and transfected in the presence of the indicated miRNAs in HEK 293 cells. Irrelevant scrambled miRNA served as a control. Cells were also co-transfected with the Renilla luciferase construct, pRL-TK. Luciferase production was measured first for the Firefly luciferase followed by the Renilla luciferase from the culture supernatant after 24 h. Firefly luciferase was normalized to the Renilla luciferase. Shown is the average from 8 independent experiments ± SD (0.0021). * p<0.001 WT DNMT1 compared to vector only control for the specified miRNA.
3. Discussion

In this study, we investigated mechanisms affecting the expression of the miRNA cluster in IPF. The six miRNAs from the *miR-17~92* cluster are transcribed together as a single pre-miRNA by a single promoter then further processed. Since we observed a decrease in each of the miRNAs in mild and moderate disease, this suggested that transcriptional regulation of the promoter for the cluster may be altered in ILD/IPF lung tissue. This promoter contains binding sites for the c-MYC transcription factor (Sawera et. al., 2005; Diosdado et. al., 2009; Hayashita et. al., 2005). Since we failed to detect changes in the expression of c-MYC, we hypothesized that other regulatory mechanisms were involved.

Examination of the upstream promoter region the *miR-17~92* cluster uncovered a region rich in CpG islands. We therefore predicted that epigenetic regulation of the cluster may be altered in ILD/IPF. Indeed, we found epigenetic silencing of the *miR-17~92* cluster in lung tissue from ILD/IPF patients compared to normal lung tissue. This silencing may increase in later stages of ILD/IPF. We also observed similar epigenetic changes in the methylation pattern in lung fibroblasts derived from patients with IPF.

The chemotherapeutic drug 5'-aza-2'-deoxycytidine is widely used in cervical cancer (Veena et. al., 2008), colorectal cancer (Flanagan et. al., 2004) gastric cancer (TO KF et. al., 2004) and prostate cancer (Huanghui et. al., 2009). This drug has been reported to alter DNA methyltion of SOCS1, lactate
dehydrogenase C, and the putative colorectal tumor-suppressor genes FLJ23749 and KIAA1456 (Flanagan et. al., 2004).

Treatment of the IPF fibroblast cell line with this drug resulted in the increase expression of the majority of the miRNAs contained in the miR-17~92 cluster and concurrent decrease in expression of predicted gene targets. After analyzing the promoter region of the IPF candidate genes, we found that some of those genes have a single or multiple CpG islands. Surprisingly, after treating the IPF fibroblasts with 5'-aza-2'-deoxycytidine, we noticed a reduced expression of the mRNA level of most of those genes such as CTGF, VEGF, MMP7, MMP9 and COL13a.

Interestingly, we did not observe changes in miR-92a in cells treated with 5'-aza-2'-deoxycytidine. This observation indicated that other regulatory mechanisms are involved in the regulation of this miRNA. Recently, a family of RNA binding proteins called hnRNPs are reported to bind miRNAs to prevent degradation and facilitate Drosha-mediated processing (Nielsen et. al., 2007; Gough et. al., 2010; Guil et. al., 2007). It has been shown that hnRNP-A1 facilitates specifically the processing of miR-18a in a context-dependent manner. This function was supported by ensuring the protection of the secondary structure for DGCR8 and Drosha recognition and/or preventing the binding of some unknown inhibitory factor (Guil et. al., 2007). Thus, it is likely that a similar mechanism is involved in the secondary regulation of the miRNAs once they are transcribed. We are currently investigating whether this possibility exists for the miR-17~92 cluster and whether changes in this process occur in ILD/IPF.
IPF involves repetitive abnormal repair of lung injuries that alter the expression of many genes (Magro et. al., 2006; Maher et. al., 2007). Furthermore, DNMT-1 but not DNMT-3a or DNMT-3b is known to be recruited to the site of DNA damage where it functions to restore epigenetic regulation (Cuozzo et. al., 2007; Le Gac et. al., 2006; Mortusewicz et. al., 2005). Our results support an important role for DNMT1 in IPF, as its expression was highest in patients with severe IPF.

Recent studies show a role for miRNA in regulating the expression of DNMTs in human cancer (Esteller et. al., 2008; Kim et. al., 2006; Fabbri et. al., 2007; Lujambio et. al., 2007). Our data shows that the miR-17-92 cluster, specifically miR-17, miR-19b, miR-20a and miR-92a, targets DNMT-1. Expressing these targets could reduce DNA methylation of the promoter for the miR-17-92 cluster. Although miR-19a has the same seed sequence as miR-19b, it did not bind to DNMT-1 as strong as miR-19b due to differences in the 3’ ends of the miRNAs that would be needed for a 3’ compensatory site with a 6-mer seed to function.

Nevertheless, whether down-regulation of DNMT-1 by enforced expression of the miRNAs or by 5’-aza-2’-deoxycytidine treatment sufficed in controlling methylation in IPF cells/tissue are crucial to attain such an effect remains to be fully elucidated. Thus, we found that a specific member of the cluster promotes DNA hypomethylation by targeting DNMT-1.

To test the role of methylation in lung fibrosis, we need to turn to a bleomycin-induced, murine pulmonary fibrosis model. Using 5’-aza-2’-deoxycytidine
concomitant with repeated intra-peritoneal bleomycin injections will be our next step in understanding IPF disease at the molecular level.

4. Conclusion

Based on our study in this chapter, we found that the CpG island in the promoter of \textit{miR-17-92} cluster is hypermethylated in IPF tissue samples and IPF fibroblasts compared to control lung tissue, where the \textit{miR-17-92} cluster is unmethylated. This methylation cause repression of the cluster expression and thus elevates the expression of cluster targets genes. 5'-aza-2'-deoxycytidine treatment up-regulates cluster expression and eliminates the methylation pressure on the CpG island of the cluster in IPF. Correspondingly, the mRNA expression of fibrotic genes was decreased upon DNA methylation inhibition, which suggests that \textit{miR-17-92} clusters may function as a fibrosis suppressor. Also, we found a novel role of \textit{miR-17-92} cluster where we showed its feedback mechanism to control its own expression through direct target to \textit{DNMT-1} 3'UTR.

Although the \textit{miR-17-92} cluster has one promoter and its own ORF (open reading frame), the individual \textit{miRNA} expression are different. Indeed, we noticed differences in expression of each individual \textit{miRNA} of the cluster. Beside, according to the seed sequence of each \textit{miRNA}, we found some similarity and differences in the target genes. This encourages us to further investigate the effect and role of each \textit{miRNA} of the cluster in our lung fibroblast cell lines to narrow our focus to a smaller number of miRNAs in the cluster. In the next
chapter, we took a further step in understanding the role on the cluster in IPF disease and its epigenetic regulation.
1- Introduction

In cancer, the main epigenetic alterations involve: distribution of histone modification patterns, aberrant DNA hypermethylation of tumor suppressor genes, and global genomic hypomethylation (Esteller et. al., 2007). The miRNA genes are no exception and follow the same epigenetic regulations. One of these, miR-127, is found surrounded by CpG islands that causes it to be repressed in normal, and not cancer, cells due to epigenetic silencing (Saito et. al., 2006) and by treating normal cells with 5'-aza-2'-deoxycytidine, high levels of miR-127 were expressed. It is worth mentioning that the miR-127 gene is located within a cluster on chromosome 14q32.31 (Altuvia et. al., 2005) and that the expression of miR-127 and miR-136 has different expression patterns than the rest of the cluster. In this study, only miR-127 is under epigenetic regulation since it is the only miRNA that was located within a CpG island (Iorio et. al., 2005; Lu et. al., 2005; Saito et. al., 2006).

In lung chronic inflammatory disease, it is recognized that many cells undergo reversible switching between a contractile phenotype and a more immature,
proliferative, synthetic or secretory phenotype (Hirst et. al., 2006; Halayko et. al., 1999). The molecular regulation, miRNA expression, and epigenetic modification for this phenotypic plasticity have not been determined.

In IPF fibroblasts, we noticed changes at a phenotypic level as well a molecular level after introducing the miR-17~92 cluster back into these cells. In addition, we and others observed that the expression of each miRNA within the cluster is not the same, suggesting a different regulatory mechanism for each individual miRNA in the cluster, even if the whole cluster is driven by a single promoter. We also showed that in IPF, the miR-17~92 cluster is methylated and repressed, and this aberrant methylation can be reversed by the epigenetic drug 5'-aza-2'-deoxycytidine, which restored most if not all of the cluster’s miRNAs expression. 5'-aza-2'-deoxycytidine was able to reverse the IPF fibroblast phenotype/genotype as well.

These findings encourage us to examine individual miRNA expression of the cluster, regulation of each miRNA, and the potential epigenetic regulation that may play a role in IPF prognosis.

2-Results

A- miR-19b and miR-20a expression affect the phenotypic characteristics of IPF lung fibroblasts

Previously, when we over-expressed the miR-17~92 cluster, we noticed changes in cell morphology. The IPF fibroblasts transfected with the cluster lost filopedia and transformed back into a spindle shape, similar to that of normal lung
fibroblasts. These results prompted us to test which single miRNA was responsible for that effect. By re-introducing individual miRNAs to IPF lung fibroblasts, we determined that miR-19b and miR-20a were able to alter the IPF fibroblast phenotype to look like normal lung fibroblasts. At the same time, inhibiting miR-19b and miR-20a expression by specific anti-miRNAs in normal lung fibroblasts induced the cells to form filopedia, resembling IPF fibroblasts (Figure 38). The remaining members of the cluster failed to induce any phenotypic changes compared to untreated cells, mock-transfected cells, or cells transfected with scrambled miRNA.

Figure 38. Effect of miR-19b and miR-20a on the morphology of lung fibroblast cell lines. Transient transfection of miR-19b and miR-20a into lung fibroblasts only changes the morphology of IPF fibroblasts. KI: knock in; KD: knock down
B-Manipulation of *miR-19b* and *miR-20a* affect senescence in lung fibroblasts

To investigate the role of *miR-19b* and *miR-20a* on lung fibroblast senescence, transfection of mature *miRNA* and antagomir *miRNA* of both *miR-19b* and *miR-20a* was performed. IPF fibroblasts showed a significant decrease in senescence after re-introducing *miR-19b* and *miR-20a*. On the other hand, further knock-down (KD) of both *miRNAs* enhanced the senescence remarkably. We also observed that the cells appeared rounded and unhealthy morphologies, with a lot of cellular debris in the media, slow cell dividing rate, ~ 20% viability by trypan blue exclusion and decline in cell density. This result was compared to mock-transfected cells and cells transfected with scrambled *miRNA* (Figure 39).
Figure 39. miR-19b and miR-20a modulate senescence in human lung fibroblasts. β-gal senescence staining after knock in (KI) and knock down (KD) of miR-19b and miR-20a in A. normal lung fibroblasts. B. IPF lung fibroblasts.
C-Knockdown of miR-19b or miR-20a decreases the in vitro proliferation of lung fibroblasts

Trypan blue dye exclusion assay and MTT proliferation assay confirmed the role of miR-19b and miR-20a in cell-cycle progression, where over-expression of both miRNAs increases the proliferation rate of normal and IPF fibroblasts. At the same time, downregulation of their expression decreased the proliferation rate, and decreased the number of viable fibroblasts as measured by Trypan Blue, which supports the senescence data. To study the proliferation rate, a scratch assay was performed 24 hours after knocking down the two miRNAs and also after re-introducing them to lung fibroblasts. (Figure40).

Figure 40. Enhancement of cell growth and proliferation by miR-19b and miR-20a in human lung fibroblasts. Antagomir -19b and -20a decrease cell proliferation while mature miRNA increased it as measured in A. MTT assay as previously described and by B. scratch assay. KI: knock in; KD: knock down ± SD (n=3. p<0.002). Data compared to mock and scrambled transfection.
Figure 40. Continued

**IPF Fibroblast**

<table>
<thead>
<tr>
<th>miR-19b KO</th>
<th>miR-19b KI</th>
<th>miR-20a KO</th>
<th>miR-20a KI</th>
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<tr>
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<td>day2</td>
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**Normal Fibroblast**

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<th>miR-19b KI</th>
<th>miR-20a KO</th>
<th>miR-20a KI</th>
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<tbody>
<tr>
<td>day0</td>
<td>day1</td>
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D-

miR19b and miR20a modulate post-transcriptional gene regulation

To further understand the role of miR-19b and miR-20a in the cluster, we transfected human lung fibroblasts with miR-19b and miR-20a separately and quantified the expression level of genes of interest (CTGF, VEGF, Ets-1, Ets-2, Elk-3, TSP-1, TERT, Col-1a and Col13a) and compared them to mock- and scrambled-transfected cells. Interestingly, re-introducing both miRNAs mimicked the effect of the entire cluster on the expression of these genes. On the other hand, knocking down miR-19b and miR-20a strongly repressed those genes.

It is worth mentioning that knocking down or over-expressing miR-19b and/or miR-20a has a significant effect on gene expression via targeting different regulatory machinery such as epigenetic regulation/methylation, which is known to regulate various genes in an efficient manner (Figure 41).
Figure 41 (A-E). Real-Time PCR results of multiple fibrotic genes after re-introduction or knocking down miR-19b or miR-20a in human lung fibroblasts. Using SAbiosciences RT-PCR primers normalized against CAP and GAPDH internal control. KI: knock in; KD: knock down
N=3, * P< 0.002
Figure 41. Continued

![Graph showing relative copy numbers of ELK-3 and TSP-1 in normal and IPF samples with various treatments.](image1)

![Graph showing relative copy numbers of Ets-1 and Ets-2 in normal and IPF samples with various treatments.](image2)

Continued
E- Epigenetic role of miR-19b and miR-20a in human IPF fibroblasts

Earlier, we co-transfected Hek293 cells with a GFP vector and 3’UTR vector accompanied with individual members of the miR-17~92 cluster or scrambled oligonucleotides. A striking decrease in luciferase expression was observed in cells transfected with miR-17, miR-19b, miR-20a and miR-92a. Surprisingly, we detected changes in both phenotype and gene expression in fibroblasts by manipulation of miR-19b and miR-20a only, and not by the other miRNAs of the cluster. These results showed semi-epigenetic global effects of miR-19b and miR-20a on lung fibroblasts. Thus, we tested the expression of DNMTs after manipulating miR-19b and miR-20a in lung fibroblasts. Further, we asked whether miR-19b and miR-20a could reverse the methylation pattern of the cluster CpG islands through a negative feedback mechanism. Interestingly, we
found that re-introducing *miR-19b* and *miR-20a* into both normal and IPF fibroblasts decreased the expression of *DNMT-1* and *DNMT-3b*, while *DNMT-3a* remained unchanged (Figure 42). This reintroduction of *miR-19b* or *miR-20a* into IPF fibroblasts restored the normal pattern of *DNMT-1* and *DNMT-3b* expression and demonstrated that there is an inverse relationship between *DNMT-1* and *miR-19b/miR-20a*.

It is worth mentioning that we did not see any changes in any *DNMT* expression by transfecting the rest of the individual *miRNAs* in the cluster.
Figure 42 (A and B). Effect of miR-19b and miR-20a on the expression of DNMTs in A. normal lung fibroblasts and B. IPF lung fibroblasts. qRT-PCR was used to measure the expression of the DNMTs in human lung fibroblasts after over expressing the cluster. Shown is the average relative expression number (RCN) normalized to CAP internal control. KI: knock in; KD: knock down N=3. *P <0.004.
**F-miR-19b and miR-20a specify the miR-17~92 cluster negative feedback regulation on its own promoter**

Virginie Olive (2009) suggests that miR-19 is the key oncogenic miRNA of the miR-17~92 cluster through activation of Akt-mTOR pathway in solid tumors. Earlier we proposed that the miR-17~92 cluster targets the 3'UTR of DNMT-1 and represses its expression, which also acted as a negative feedback mechanism to regulate its own promoter. In this chapter we explore the precise role of the cluster in IPF epigenetic regulation and show that miR-19b and miR-20a induce a potent anti-fibrotic activity in IPF fibroblast cell lines. We suggest that miR-19b and miR-20a are the key players in the feedback role of the miR-17~92 cluster. Then, we studied the methylation pattern of the miR-17~92 CpG island after manipulating miR-19b and miR-20a expression in lung fibroblast cell lines. Consistent with our previous data, re-introducing miR-19b and miR-20a into IPF fibroblasts significantly decreased the methylation of the CpG islands.

On the other hand, further knocking down of miR-19b shifted the methylation pattern to be more hypermethylated (60%) compared to untreated cells, mock-transfected cells, and cells transfected with scrambled oligonucleotides. Notably, knock-down of miR-19b had a stronger effect on switching the methylation pattern toward hypermethylation than miR-20a (Figure 43).
Figure 43. Effect of miR-19b and miR-20a on the DNA methylation of its promoter lung fibroblasts. DNA was isolated from lung fibroblasts using Methyl-Profiler™ DNA Methylation qPCR Primer assay. The average percent of unmethylated DNA to methylated DNA for the miR-17~92 was determined. KI: knock in; KD: knock down *P<0.003, N =3

3-Discussion

We explored the role of the miR-17~92 cluster in IPF patient lung samples and lung fibroblast cell lines derived from IPF patients in which the cluster was down-regulated in IPF compared to normal. We demonstrated that in IPF, the miR-17~92 cluster is decreased with increasing severity of the disease. In addition, we investigated the epigenetic regulation of the cluster that caused it to be repressed in IPF while methylation is increased. Recently, IPF disease has started to be classified as an aging disorder that affects older adults (Castriotta et. al., 2010), although others have shown that the miR-17~92 cluster, especially miR-17, miR-19b, miR-20a and miR-106a/miR-17, are down regulated in human aging (Hackl et. al., 2010). These finding support and are consistent with our
work, particularly when we correlate IPF versus the miR-17~92 cluster in order to get a better understanding of IPF, molecularly.

Recent studies revealed the importance of the whole cluster in cancer, angiogenesis, and development (He et. al., 2005; Hayashita et. al. 2005; Ventura et. al., 2008; Lu et. al., 2005; Dews et. al., 2006; O'Donnell et. al., 2005). Others showed the role of some of the individual members of the miR-17~92 cluster in different diseases. For example, Virginia Olive (2009) revealed the oncogenetic activity of miR-19b. Another study showed that miR-17 over-expression reduces cell proliferation, migration, and adhesion in mice, suggesting that miR-17 may regulate the same pathway positively and negatively to reach homeostasis (Shan et. al., 2009). These results support our findings when we test the functionality of the miR-17~92 cluster in IPF at the molecular level and study the regulation effect of each individual miRNA on epigenetic and gene expression. Not surprising, we found that miR-19b and miR-20a are the crucial players of the cluster. Re-expressing miR-19b or miR-20a in IPF lung fibroblast was enough to change the morphology of IPF fibroblasts into the spindle shapes characteristic of normal fibroblasts. It was also able to increase the proliferation of lung fibroblasts and decrease senescence. In addition, re-introduction of miR-19b and miR-20a in IPF reduces the expression of many fibrotic and angiogenic genes (CTGF, VEGF, Ets-1, Ets2, Elk-3, TSP-1, Col-1a and Col13a) and at the same time decreases DNMT-1 and DNMT-3b expression in normal and IPF fibroblast. Furthermore, we found the opposite effect when miR-19b and miR-20a were knocked down by an antagonir against each miRNA. Interestingly, we did not
detect any changes or alteration in phenotype nor on gene expression when we studied the rest of the miRNAs in the cluster, which shows the high specificity and accuracy of our results.

In the previous chapter, we validated the miRNA-DNMT-1 interaction using a firefly luciferase assay, where we showed that four of six members of the miR17~92 cluster significantly reduced the luciferase expression of the wild type 3'UTR of DNMT-1 with respect to mock and scrambled oligonucleotides. On the other hand, we mutated DNMT-1 3'UTR where the miRNA seed sequence binds, and used it as a control to test the specificity of the experiment. We did not detect any significant changes on luciferase activity using the mutated DNMT-1 3'UTR.

To assess the ability of those miRNAs to down-regulate endogenous DNMT-1, we over-expressed or knocked down the miR-19b and miR-20a in fibroblasts then performed real-time RT-PCR using RNA from those cells and compared mRNA levels of DNMTs to levels in mock-transfected cells, cells transfected with scrambled oligonucleotides, and cells transfected with other miRNAs from the cluster.

Overexpression of miR-19b or miR-20a induced a marked reduction of DNMT-1 at the mRNA level. On the other hand, knocking down miR-19b or miR-20a with antagonir up-regulated DNMT-1. We did not detect any changes in DNMT-3a after manipulating both miRNAs in fibroblasts.

This observation suggests that miR-19b and miR-20a may contribute to DNA epigenetic regulation in IPF as well as affect the methylation pattern of the miR-17~92 CpG island - causing its repression in IPF. Indeed, we found that the
methylation pattern of the cluster CpG island was changed from being 100% intermediately-methylated to being mostly unmethylated when we knocked down \textit{miR-19b} and \textit{miR-20a}. Alternatively, the further knocking down of \textit{miR-19b} or \textit{miR-20a} caused a significant increase in hypermethylation of the cluster CpG island and nearly abolished its intermediate methylation pattern.

\textbf{4-Conclusion}

Our data suggest that regulation of \textit{DNMT-1} through \textit{miR-19b} and \textit{miR-20a} of the cluster may act as a sensor of DNA methylation capacity in IPF. To test the role of methylation in lung fibrosis, we will utilize the bleomycin-induced murine pulmonary fibrosis model. Using 5'-aza-2'-deoxycytidine concomitant with repeated intra-peritoneal bleomycin injections will be our next step in understanding IPF disease at the molecular level.
CHAPTER SEVEN: 5’-AZA-2’-DEOXYCYTIDINE AS A POTENTIAL CHEMOTHERAPEUTIC DRUG IN IDIOPATHIC PULMONARY FIBROSIS (IPF)

1-Introduction

Pulmonary fibrosis is a progressive disorder that shows severe fibrotic molecular pathology leading to restriction of lung function and death (Katsuma et al., 2001; Selman et al., 2003). As mentioned in previous chapters, IPF occurs after repetitive inflamed injuries ending with abnormal fibrotic repair (Selman et al., 1996). Current therapies have focused on preventing lung inflammation more than fibrosis, which has not been proven to alleviate disease. Until today, no therapy has been effective to treat ILD/IPF patients. Understanding the fundamental mechanisms that regulate ILD/IPF progression will lead to effective and specific treatments. Much of the information concerning ILD/IPF mechanisms comes from studies of the widespread classical and accepted model of bleomycin-induced lung injury and fibrosis in animals. At the same time, the relevance to human lung fibrosis is still unclear (Kaminski et al., 2003).

Bleomycin is a cytotoxic drug that was originally isolated from *Streptomyces verticillatus* (Umezawa et al., 1967). It causes DNA strand breakage (Lown et al., 1977) and oxidant injury (Tanaka et al., 2010). Bleomycin was initially
considered as a therapy against squamous cell carcinomas (Umezawa et. al., 1974), however, its effectiveness was limited by dose-dependent pulmonary toxicity that induced lung inflammation in a similar manner to pulmonary fibrosis, followed by progressive destruction of normal lung architecture. These disease processes were also observed in many animal models after intrapritoneal (IP), intravenous (IV), subcutaneous (SC), or intratracheal (IT) delivery of bleomycin (Kaminski et. al., 2003; Katsuma et. al., 2001; Tran et. al., 1997; Elias et. al., 1990; Muggia et. al., 1983).

The pathology and the histology of bleomycin-induced pulmonary fibrosis has been well characterized and includes signs of severe lung injury including leakage of fluid and plasma protein into the alveolar space, damage to alveolar epithelium, and development of hyaline membrane (Moor et. al., 2007). On the other hand, the regulation of bleomycin-induced lung injury at the molecular level is still unclear. Many studies showed that in bleomycin-treated mice, the fibrotic response to bleomycin is strain-dependant, where C57Bl/6 mice are more vulnerable to fibrosis than Balb/c mice (Harrison et. al., 1987; Schrier et. al., 1983) due to differences in expression of bleomycin hydrolase, the inactivating enzyme (Sebti et. al., 1989).
2-Characterization of the bleomycin-induced pulmonary fibrosis model

Many studies showed that the body weight of mice treated with bleomycin is reduced during the first 5 days after injection. In our study, the lung weight of those mice treated with bleomycin was less than controls (Figure 44A, B). At the same time, progressive fibrosis was evident in bleomycin-treated mice compared to controls, which had no visible lesions (Katsuma et. al., 2001). Furthermore, at day 2 after bleomycin treatment, the collagen deposition was observed and by day 5, fibrotic changes were clearly visible with no changes in the architecture of the lung. But at 7-14 days, significant changes were observed, including increases in fibroblasts, increases in fibrotic collagens, thickening of the lung septa, and recruitment of many fibrotic cells (Figure 44C) (Katsuma et. al., 2001).
cDNA microarray performed on bleomycin-treated mice at different time points revealed four separate gene clusters. Cluster 1 includes genes which were up-regulated after 5 days of bleomycin treatment then subsequently returned to basal levels. Cluster 2 consists of genes that were up-regulated after...
5 days of bleomycin treatment and then maintained their high expression. Cluster 3 includes genes that were induced during 14 days of the experiment. Finally, cluster 4 contains genes that were repressed after day 2 or day 5 of treatment (Figure 45) (Katsuma et. al., 2001). Similar results were obtained using an oligonucleotides microarray (Kaminski et. al., 2000). Both data sets demonstrate that bleomycin treatment caused changes in the inflammatory and molecular response that correlated with histopathological changes (Katsuma et. al., 2001; Kaminski et. al., 2000).
Figure 45. Cluster analysis of differentially-expressed genes identified by cDNA microarray-based gene expression profiling. One hundred and fifty-nine clones (representing 82 non-redundant genes) derived from cDNA microarray analysis were differentially-expressed (over twofold differences) in three independent experiments. These genes were classified into four clusters using a k-means method. Each graph shows a time course of expression data for genes in each cluster at days 2, 5, 7, and 14 after bleomycin injection. Values for each gene at each time point are plotted as mean ratios ($n = 3$) (Katsuma et. al., 2001). Yellow color indicates the highly expressed genes while gray/ light blue color indicates low expressed genes.
3-Results

A-Down regulation of \textit{miR-17-92} cluster in bleomycin-treated mice

Our previous study of IPF showed that \textit{miR-17-92} cluster was down-regulated due to methylation repression. Thus it is worth studying these results in a murine model of IPF (bleomycin-induced lung injury and fibrosis in mice).

Bleomycin given twice weekly by intra-peritoneal injections for four weeks results in sub-pleural fibrosis similar to human pathology (Baran et. al., 2007). We have observed that inflammation and fibrosis is visually apparent after two weeks of treatment. Therefore, to mimic a mild disease state, we treated C57/BL6 mice four weeks with bleomycin and total lung RNA was isolated to analyze the
expression of the *miR-17~92* cluster. As shown in Figure 46, treatment with bleomycin alone resulted in decreased expression of the *miR-17~92* cluster and increased expression of *miR-17~92* target genes at both the mRNA and protein level, compared with saline treated controls mice. This result gives us a promising model of IPF since it has similar *miR-17~92* expressions as human IPF.
Figure 46. MiR-17~92 cluster is repressed in bleomycin-treated mice while its target genes are highly expressed. A. qRT-PCR was performed. Shown is the average relative expression normalized to Sno-202 internal control ± S.E.M.(0.0032) B. Gene expression was assayed using RT-PCR and normalized to GAPDH and CAP genes± S.E.M.(0.0032) C. Western blot analysis was performed using specific antibodies to determine the expression of some fibrotic genes at protein levels in bleomycin-treated mice/IPF murine model. β-actin was used as the loading control. N=8 *P<0.0014.
Knowing that in bleomycin-treated mice the miR-17~92 cluster has the same repressed expression in both IPF and its murine model of bleomycin-induced pulmonary fibrosis, we attempted to elucidate the reason for this repression in IPF and confirmed it in this model. As predicted, we found that the CpG island of the cluster was methylated in bleomycin-treated mice compared to normal saline-treated controls (Figure 47). Others have shown that in bleomycin-treated mice, other anti-fibrotic genes were repressed epigenetically, especially by DNA methylation and deacetylation of histones (Huang et. al., 2009). In addition, it was shown in lung fibroblasts from a mouse model of pulmonary fibrosis and IPF patients that global DNA methylation is increased and concomitant DNMTs were
highly-expressed through activation of PTEN and inhibition of Akt signaling (Huang et. al., 2010). Furthermore, other studies showed that DNMTs (especially $DNMT-1$) recruitment is increased in bleomycin-treated mice to store and repair the DNA damage and degradation caused by bleomycin treatment (Leng et. al., 2008; Palii et. al., 2008). These results support our findings and suggest that DNA methylation plays a crucial role in the development of lung fibrosis in bleomycin-treated mice.

Figure 47. DNA methylation of $miR-17$–$92$ CpG promote in mouse model of pulmonary fibrosis. DNA was isolated from lung bleomycin-treated mice. Using Methyl-Profiler™ DNA Methylation qPCR Primer assay, the average percent of unmethylated DNA to methylated DNA for the $miR-17$–$92$ was determined. KI: knock in; KD: knock down *$P<0.003$, $N=3$. 

\begin{figure}
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\includegraphics[width=0.5\textwidth]{figure47.png}
\caption{DNA methylation of $miR-17$–$92$ CpG promote in mouse model of pulmonary fibrosis. DNA was isolated from lung bleomycin-treated mice. Using Methyl-Profiler™ DNA Methylation qPCR Primer assay, the average percent of unmethylated DNA to methylated DNA for the $miR-17$–$92$ was determined. KI: knock in; KD: knock down *$P<0.003$, $N=3$.}
\end{figure}
C- 5’-aza-2’-deoxycytidine induces the expression of the \textit{miR-17~92} cluster \textit{in vivo}

Since 5’-aza-2’-deoxycytidine is used clinically, we examined the doses tolerated in mice in preliminary studies. We treated mice weekly with 0.156, 0.3125, 0.625, or 1.25 mg/kg of 5’-aza-2’-deoxycytidine for a total of four weeks. We did not encounter any noticeable toxicity, abnormal inflammation or fibrosis in the mice nor did we observe any deaths in the treated mice. After four weeks, we examined \textit{miR-17~92} expression in the lung by qRT-PCR and found that it was increased in the treated mice.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure48.png}
\caption{In vivo treatment of mice with different concentration of 5’-aza-2’-deoxycytidine induces the expression of the \textit{miR-17~92} cluster in the lung. Mice were treated with 0.156, 0.3125, 0.625, or 1.25 mg/kg/week of 5’-aza-2’-deoxycytidine i.p. for 4 weeks. RNA was extracted from the lung tissue and miRNA expression was examined by qRT-PCR. Shown is the average relative expression \pm SD (0.0028), \(n=4\) mice per treatment group. *\(p<0.002\) for PBS vs 4 week 5’-Aza for all miRNAs.}
\end{figure}
Since the mice tolerated the 5'-aza-2'-deoxycytidine treatment, we next treated the mice with the lowest dose (0.156 mg/kg/week) to determine the time frame in which we would observe changes in the expression of the miR-17~92 cluster. As shown in Figure 49, we observed a modest increase in miRNA expression in mice treated with 5'-aza-2'-deoxycytidine for two weeks. However, we observed a better response in the induction of the miRNA expression after four weeks of treatment with 5'-aza-2'-deoxycytidine.

![Figure 49](image)

**Figure 49.** *In vivo* treatment of mice with 5'-aza-2'-deoxycytidine induces the expression of the miR-17~92 cluster in the lung. Mice were treated with 0.156 mg/kg/week of 5'-aza-2'-deoxycytidine i.p. for four weeks. RNA was extracted from the lung tissue and miRNA expression was examined by qRT-PCR. Shown is the average relative expression ± SD (0.0041), n=4 mice per treatment group. P<0.002 for PBS vs 4 week 5'-Aza for all miRNAs.
D-5'-aza-2'-deoxycytidine alters gene expression in murine model of pulmonary fibrosis

Since 5'-aza-2'-deoxycytidine altered DNA methylation patterns leading to the restoration of miR-17~92 expression and ultimately decreased expression of DNMTs and fibrotic genes in IPF lung fibroblasts, we next evaluated whether we could see similar effects in a murine model of pulmonary fibrosis. We treated C57/BL6 mice with bleomycin for two weeks prior to treatment with 5'-aza-2'-deoxycytidine. We also treated mice concurrently with both bleomycin and 5'-aza-2'-deoxycytidine.

<table>
<thead>
<tr>
<th>week 1 &amp; 2</th>
<th>week 3 &amp; 4</th>
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<tr>
<td>PBS control</td>
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<td>Bleomycin control</td>
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<tr>
<td>5'-Aza treatment only</td>
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<td>5'-Aza treatment only</td>
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<tr>
<td>bleomycin and 5'-Aza treatment</td>
<td>bleomycin only</td>
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As shown in Figure 50A, treatment with bleomycin alone decreased the expression of the miR-17~92 cluster in the lungs. Nevertheless, treatment with 5'-aza-2'-deoxycytidine after bleomycin prevented the cluster from being repressed. In addition, after treating mice with bleomycin, we found that the chemotherapeutic drug 5'-aza-2'-deoxycytidine was able to reverse the effects of
bleomycin of mice treated first. We also observed a marked decrease in collagen deposition based on quantification using trichrome stained lungs (Figure 50B).

Figure 50. In vivo treatment of mice with 5’-aza-2’-deoxycytidine (5’AZA) induces the expression of the miR-17~92 cluster in the lung. Mice were treated with 0.156 mg/kg/week of 5’-aza-2’-deoxycytidine i.p. for four weeks. RNA was extracted from the lung tissue and miRNA expression was examined by qRT-PCR. Shown is the average relative expression ±SD (0.0025), n=4 mice per treatment group. p<0.002 for PBS vs. 4 week 5’ AZA for all miRNAs.
In mice receiving bleomycin and 5'-aza-2'-deoxycytidine, we found that fibrotic gene expression was decreased and the expression of VEGF, CTGF, Col1a, Col3a and Col13a in mice receiving bleomycin and 5'-aza-2'-deoxycytidine returned to levels comparable to those in the PBS control samples (Figure 51).

![Diagram of gene expression in bleomycin mice treated with 5'AZA](image)

**Figure 51. In vivo treatment of mice with 5'-aza-2'-deoxycytidine attenuates bleomycin-induced fibrotic gene expression.** Mice were treated as described in Figure 41. RNA was extracted from the lung tissue and miRNA expression was examined by qRTPCR. Shown is the average relative expression ±SD (0.0013), n=4 mice per treatment group.

As expected, the expression of the DNMTs was also decreased in the mice treated with both 5'-aza-2'-deoxycytidine and bleomycin (Figure 52).
Figure 52. 5’-aza-2’-deoxycytidine decreases DNMT expression in a mouse model of pulmonary fibrosis. Mice were treated as described in Figure 41. The expression of DNMTs within the lung tissue was examined by qRT-PCR. Shown is the average relative expression ± SD, n=4 mice per treatment group.

The level of DNMTs mRNA expression showed an approximate sevenfold decrease by treatment with bleomycin and 5’-aza-2’-deoxycytidine. Since an inverse relationship existed between the expression of DNMTs and the expression of the miR-17~92 cluster in the mice treated with 5’-aza-2’-deoxycytidine and bleomycin, we predicted that the DNA methylation of the cluster would also be changed. We found that treatment of the mice with 5’-aza-2’-deoxycytidine decreased the bleomycin-induced methylation of the CpG island of the miR-17~92 cluster (Figure 53).
Figure 53. 5’-aza-2’-deoxycytidined decreases DNA methylation of miR-17~92 CpG in a mouse model of pulmonary fibrosis. Mice were treated as described in Figure 41. Using Methyl-Profiler™ DNA Methylation qPCR Primer assay, the average percent of unmethylated DNA to methylated DNA for the miR-17~92 was determined. n=3, *P<0.0012.

4-Discussion

Many potential therapies have been evaluated for patients with IPF. Most of these studies have focused on the effects of corticosteroids, cytotoxic agents, or colchicine in IPF (ATS, 2000; Baughman et. al., 1992; Hunninghake et. al., 1995). To test the role of methylation in lung fibrosis, we turned to a murine model of bleomycin-induced pulmonary fibrosis. Using 5’-aza-2’-deoxycytidine concomitant with repeated intra-peritoneal bleomycin injections, we found that the demethylating agent reduced lung fibrosis and also re-stimulated the production of the miR-17~92 cluster. We are currently analyzing whether treating
animals after the fibrotic phenotype appears with 5’-aza-2’-deoxycytidine will be an effective treatment in reducing fibrosis. To our knowledge, this study is the first report showing that the miR-17~92 cluster is downregulated in IPF and re-expression of the cluster by genetic means or by using 5’-aza-2’-deoxycytidine represses expression of fibrotic genes through changes in DNA methylation of the cluster.

5-Conclusion

In our study, we are suggesting a global treatment for IPF targeting epigenetic alterations in IPF lung tissues. Using a preclinical murine model of pulmonary fibrosis, our data suggest that epigenetic mechanisms have a crucial rule in progression of the disease, and reversing this process with 5’-aza-2’-deoxycytidine appears to be a promising therapy in IPF. Combination with other epigenetic treatments or therapies specifically targeting the miRNAs may be a potential therapeutic value in the treatment of IPF patients.
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