Characterisation of the role of long non-coding RNAs in lung fibroblasts from control and idiopathic pulmonary fibrosis patients

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Characterisation of the role of long non-coding RNAs in lung fibroblasts from control and idiopathic pulmonary fibrosis patients

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Abbreviations

AECs – Alveolar epithelial cells

AGTR1 – Angiotensin II receptor type 1

APTR – Alu-mediated p21 transcriptional regulator

ATS – American Thoracic Society

BAL– Bronchoalveolar lavage

BALF – Bronchoalveolar lavage fluid

CCK-8 – Cell Counting Kit-8

CCL – Chemokine (C-C motif) ligand

Cdk6 – Cyclin-dependent kinase 6

ceRNAs – Competing endogenous RNAs

ChIP – Chromatin immunoprecipitation

ChIP-seq – Chromatin immunoprecipitation with sequencing

CHRF – Cardiac hypertrophy-related factor

circRNA – Circular non-coding RNA

ciRNA – Circular intronic RNA

CTGF – Connective tissue growth factor

CXCL – Chemokine (C-X-C motif) ligand

DLCO – Diffusing carbon monoxide

DNMT – DNA methyltransferase

DPLD – Diffuse parenchymal lung diseases

DUSP5 – Dual specificity protein phosphatase 5

ECM – Extracellular matrix

ELISA – Enzyme-linked immunosorbent assay

EMT – Epithelial-mesenchymal transition

ER – Endoplasmic reticulum

eRNA – Enhancer RNA

ERS – European Respiratory Society

FAK – Focal adhesion kinase

FBS – Fetal Bovine Serum

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

FGFR – Fibroblast growth factor receptor

FVC – Forced vital capacity

FVIIa – Activated factor VIIa

FX – Factor X

FXa – Activated factor Xa

GAS5 – Growth arrest-specific transcript 5

H3K36me3 – Histone H3 trimethylation of lysine 36

H3K4me1 – Histone H3 monomethylated at lysine 4

H3K4me3 – Histone H3 trimethylation of lysine 4

HOTAIR – HOX transcript antisense RNA

HOTTIP – HOXA Distal Transcript Antisense RNA

HRCT – High resolution computed tomography

HSC – Hepatic stellate cell

IFN-γ – Interferon gamma

IIPs – Idiopathic interstitial pneumonias

IKK – IκB kinase

IL-13 – Interleukin 13

IL-17A – Interleukin 17A

IL-1R – IL-1 receptor

IL-1β – Interleukin 1β

IL-6 – Interleukin 6

ILD – Interstitial lung disease

IPF – Idiopathic pulmonary fibrosis

IRES – Internal ribosome entry site

JAK – Janus kinase

JNK – c-Jun N-terminal kinase

JUNB – Transcription factor jun-b

LAP – Latency associated proteins

LincRNA – Long intergenic non-coding RNA

LIPCAR – Long intergenic non-coding RNA predicting cardiac remodelling

LLC – Large latent complex

LNA – Locked nucleic acid

LncRNA – Long non-coding RNA

LTBPs – Latent TGF binding proteins

MALAT1 – Metastasis associated lung adenocarcinoma transcript 1

MAPK – Mitogen-activated protein kinase

MCP – Monocyte chemoattractant protein

MEG3 – Maternally expressed gene 3

Mhrt – Myosin heavy chain-associated RNA transcript

MIAT – Myocardial infarction associated transcript

miRNA – microRNA

MLL1 – Mixed-lineage leukaemia protein 1

MMPs – Matrix metalloproteinases

mRNA – Messenger RNA

MyD88 – Myeloid differentiation factor 88

NAC – N-Acetylcysteine

NAT – Natural antisense transcript

ncRNA – Non-coding RNA

NEAT1 – Nuclear enriched abundant transcript 1

NF-κB – Nuclear factor-κB

NICE – National Institute for Health and Care Excellence

NK – Natural Killer

ORF – Open reading frame

p38α – Mitogen-activated protein kinase 14

PAI-1 – Plasminogen activator inhibitor 1

PAR1 – Proteinase-activated receptor-1

PBS – Phosphate Buffered Saline

PCR – Polymerase chain reaction

PDGF – Platelet-derived growth factor

PDGFR – Platelet-derived growth factor receptor

PFAR – Pulmonary fibrosis-associated RNA

PFT – Pulmonary function tests

PI3K – Phosphatidylinositol-3-kinase

PLCγ – Phospholipase Cγ

PRC – Polycomb repressive complexes

qRT-PCR – Quantitive Real-Time Polymerase chain reaction

RISC – RNA–induced silencing complex

RNA-seq – RNA-sequencing

RNAPII – RNA polymerase II

RNAPIII – RNA polymerase III

RNase P – Ribonuclease P

ROS – Reactive oxygen species

SFTPA2 – Surfactant protein A2

SFTPC – Surfactant protein C

SINE – Short interspersed element

SMURF2 – SMAD specific E3 ubiquitin protein ligase 2

snoRNA – Small nucleolar RNA

snoRNP – Small nucleolar ribonucleoproteins

snRNA – Small nuclear RNA

SRF – Serum response factor

STAT – Signal transducer and activator of transcription

TAK1 – Transforming growth factor beta-activated kinase

TF – Tissue Factor

TGF-β1 – Transforming growth factor β1

T_H – T-helper

TIMPs – Tissue inhibitors of metalloproteinases

TLC – Total lung capacity

TNF-α – Tumor necrosis factor α

TRAF6 – TNF receptor-associated factor 6

TβRI – TGF-β1 receptor

TβRII – TGF-β2 receptor

Uchl1-as1 – Ubiquitin carboxy-terminal hydrolase L1 antisense RNA 1

UTR – Untranslated region

UUO – Unilateral ureteral obstruction

VEGFR – Vascular endothelial growth factor receptor

XIST – X inactive specific transcript

YAP1 – Yes-associated protein 1

α-SMA – α-smooth muscle actin

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a fatal progressive chronic disease characterised by excessing scarring of the lungs leading to irreversible decline in lung function. The aetiology and pathogenesis of the disease are still unclear, although lung fibroblast activation and secretion of fibrotic and inflammatory mediators have been strongly associated with the development and progression of IPF. Significantly, long non-coding RNAs (lncRNAs) are emerging as novel modulators of multiple biological processes although their function and mechanism of action is poorly understood and their role in IPF is uncharacterised.

To better understand the underlying pathological features of IPF, the phenotypic changes of control and IPF lung fibroblasts as well as the role of lncRNAs in IPF and during TGF-β1, PDGF-AB and IL-1β-induced activation of lung fibroblasts were examined. Overall, IPF fibroblasts demonstrated an increased fibrotic and a reduced inflammatory and proliferative profile compared to controls. The phenotypic differences of control and IPF fibroblasts were also reflected at the epigenetic level. Using chromatin immunoprecipitation combined with sequencing (ChIP-seq), the distribution profile of the histone modification H3K4me1 was shown to be notably different between the two fibroblast populations.

RNA-sequencing (RNA-seq) and microarray technology identified several differentially expressed lncRNAs in lung fibroblasts upon TGF-β1 and IL-1β activation. Subsequent knockdown studies focused on the functional roles of four lncRNAs namely LINC00960, LINC01140, IL7AS and MIR3142HG. LINC01140 was found to be a negative regulator of the inflammatory response, while both LINC00960 and LINC01140 were shown to be positive regulators of proliferation. Additionally, IL7AS and MIR3142HG were also shown to regulate the inflammatory response in control and IPF fibroblasts. Collectively, the results of this thesis propose that lncRNAs may be important regulators of lung fibroblast functions and consequently mediate the progression of IPF.

1. Chapter 1 – Introduction

1.1. The respiratory system

The respiratory system is a complex and highly organised organ system consisting of multiple different cell types that are required for gas exchange i.e. the uptake of oxygen and release of carbon dioxide from the blood. Its association with disease was first acknowledged in the *Hippocratic corpus,* in which Hippocrates documents the concept of symptoms and diagnosis of pulmonary infections around the 5^{th}- 4^{th} century BC (Tsoucalas and Sgantzos, 2016). However, the function of respiratory organs appears to have been first explored by Erasistratus, a Greek physician, in 280 BC. He supposed that the *pneuma* (circulating air or 'spirit' translated from ancient Greek) could enter the body through the lungs in the process of inhalation. Although his work was challenged by Galen and other scientists centuries later, he undoubtedly laid the foundations of respiratory research (Pearce, 2013). The notion of breathing was difficult to comprehend for hundreds of years and it was therefore a topic to be challenged systematically. However, the discovery of oxygen was a breakthrough for our understanding of respiration and provided the path for continuous progress in respiratory research. Therefore, since the early investigations of lung function, modern pulmonology and scientific research has developed dramatically our knowledge of respiration, an essential human physiological process.

Indeed, we now understand that gas exchange requires the respiratory and circulatory systems to work together to transport oxygen and carbon dioxide to and from the lungs. Briefly, upon inhalation air enters the body through the nose or mouth where air rich in oxygen travels to the lungs via the pharynx and trachea which branches into the bronchi, the left and right bronchus. The bronchi serve as airway passages which enter the lungs and further divide into the bronchioles in tree-like structures. These define the end of the conducting zone and the start of the respiratory zone of the respiratory tract. The terminal structure of the respiratory bronchioles are the pulmonary alveoli which are the primary site of gas exchange in the lungs. At this anatomical structure, the alveolar ducts connect the respiratory bronchioles to the air sacs

which contain the alveoli, the smallest units of the respiratory tract. The alveoli are composed of Type I and II alveolar epithelial cells (AECs) and macrophages and are covered by capillaries. The alveolar epithelium and endothelial cells of the capillaries compose the very thin gas permeable membrane barrier, also known as the blood-air barrier, where the process of gas exchange occurs by simple diffusion. The membrane is characterised by its elasticity to allow the alveoli to stretch during inhalation and exhalation. The barrier allows oxygen carried from the alveoli to diffuse into the circulation via the capillaries and carbon dioxide out of the capillaries back to the alveoli.

The airways and alveoli are surrounded and protected by layers of tissue consisting of nearly 50 cell types (Breeze and Wheeldon, 1977). These assist with the various structural and functional needs of the respiratory area and include cells such as lung fibroblasts, epithelial, smooth muscle and inflammatory cells (Hogan et al., 2014). Maintaining homeostasis in this environment is crucial during respiration as well as response to injury and infections (Thompson et al., 1995).

The development of the respiratory system begins at embryogenesis and continues until early adolescence through controlled cell proliferation and differentiation. Although infant lungs can sustain life after birth, respiratory development is a long process which takes several years to complete (Pinkerton, 2000). The embryonic, pseudoglandular, canalicular, saccular, and alveolar stages are essential developmental stages for the growth of the fully functional and complex respiratory structures (Burri, 1984). Several studies have even shown that genetic or environmental stimuli that may interfere at any time with respiratory development result in an increased risk of lung dysfunction and disease in later life (Kajekar, 2007; Macneal and Schwartz, 2012).

1.1.1. Interstitial lung diseases

Interstitial lung diseases (ILD), more specifically known as heterogeneous diffuse parenchymal lung diseases (DPLD), are a group of heterogeneous lung diseases which share similar clinical and pathophysiological features. ILDs are a large group of mostly chronic but also acute lung diseases characterised by variable degrees of inflammation and fibrosis of the lung interstitium (Meyer et al., 2012). The cause of ILDs can be either identifiable or unknown, as in the case of idiopathic pulmonary fibrosis (IPF) (American Thoracic Society European Respiratory Society, 2002). Exposure to fumes, dust, radiation, microbes and other occupational and environmental allergens or hazards have been identified as triggers for the development of ILDs. Additionally, granulomatous inflammation as well as autoimmune and genetic diseases have also been found to play a role in the development of ILDs (Mikolasch et al., 2017).

Over a hundred types of ILDs have been described, each one displaying an array of unique pathophysiological processes. The complexity of these diseases and their tendency to display similar phenotypes means distinguishing between the different types of ILDs can be challenging (American Thoracic Society European Respiratory Society, 2002). Therefore, a robust collection of physical and medical tests as well as a detailed medical history are important to establish an accurate diagnosis. Successful management of ILDs is very much diagnosis-dependent, as different types of ILDs require specific and targeted therapeutic approaches (Meyer, 2014).

IPF is considered to be the most common of the ILDs, and it more accurately fits into the sub-group of idiopathic interstitial pneumonias (IIPs) (Raghu et al., 2011; Kekevian et al., 2014). The National Institute for Health and Care Excellence (NICE) regularly updates its guidelines for respiratory physicians and other professionals as well as information and recommendations to the public. The guidelines are updated according to new clinical and research evidence which are summarised in these documents. The American Thoracic Society (ATS) and the European Respiratory Society (ERS) also provide

official guidelines for the diagnosis and management of IIPs (Meyer et al., 2012; Travis et al., 2013; American Thoracic Society European Respiratory Society, 2002).

Over the past decades, IPF has garnered increasing attention within the clinical and research sphere in light of significantly increased incidence. Mortality and morbidity rates in the United States have been on the rise between 1992 and 2003 (American Thoracic Society European Respiratory Society, 2002; Olson et al., 2007). Likewise, IPF cases have doubled in the UK between 1990 and 2003 and evidence suggests that incidence will continue to increase in the future (Gribbin et al., 2006; Lee et al., 2014).

1.2. Idiopathic pulmonary fibrosis

Fibrosis is a pathophysiological condition that can affect nearly every organ in the human body where irregular and excessive accumulation of scar tissue leads to organ failure and potentially death as seen in the final stages of fibrotic diseases such as pulmonary (King et al., 2011), cardiac (Kong et al., 2014), nephrotic (Duffield, 2014) and hepatic fibrosis (Bataller and Brenner, 2005). In combination with genetic factors, tissue injuries may provoke the development of fibrosis including exposure to damaging environmental stimuli such as irritants, smoke, radiation, viral and bacterial infections (Raghu et al., 2011; Macneal and Schwartz, 2012).

IPF is a progressive chronic ILD which is characterised by scar tissue accumulation and therefore thickening of the normal lung walls, leading to impaired gas exchange and restricted ventilation. IPF is a disease of unknown aetiology, making development of effective drug treatments particularly challenging (Raghu et al., 2011). Nonetheless, scientists have been intensively researching the molecular and cellular mechanisms of the disease and although the pathogenesis of IPF is still unclear, several theories regarding the pathophysiology of IPF have been proposed (Todd et al., 2012).

As is the case with most ILDs, inflammation was initially thought to be the major player in IPF until unresponsiveness to anti-inflammatory medications prompted the re-evaluation of this idiom (Richeldi et al., 2003; Davies et al., 2003). However, the presence of immune cells in IPF lungs has been a consistent pathological finding and may be important in the development of the disease (King et al., 2001b; Gross and Hunninghake, 2001; Katzenstein and Myers, 1998; Balestro et al., 2016; Travis et al., 2013).

The histology of fibrotic lungs indicates irreversible accumulation of scarred tissue characterised by collagen deposition and other alterations to the extracellular matrix (ECM) which dramatically remodels the lung architecture by stiffening the distal airspaces and parenchyma (Raghu et al., 2011). It has been suggested that lung fibrosis could be provoked by a number of different cell types including epithelial cells, fibroblasts, myofibroblasts and immune cells (King et al., 2011).

1.2.1. Epidemiology

It has been challenging to accurately assess the incidence and prevalence of IPF as there is limited epidemiological data on the disease, an issue that is compounded by the regular changes to the diagnostic criteria. As such, estimates vary as a result of demographic differences, study population and the case definition used to identify patients with IPF (Nalysnyk et al., 2012).

Studies in the United States (Raghu et al., 2016), Canada (Hopkins et al., 2016) and Europe, including Greece (Karakatsani et al., 2009), Finland (Hodgson, 2002), Norway (Plessen et al., 2003) and the United Kingdom (Gribbin et al., 2006; Navaratnam et al., 2011), have reported widely different incidence and prevalence of IPF. Interestingly, a meta-analysis of 34 studies by Hutchinson *et al*. (Hutchinson et al., 2015) suggests that incidence of IPF is currently comparable to malignancies such as testicular, stomach liver and cervical cancers. A systematic review by Nalysnyk *et al*. analysed 15 studies of the incidence and prevalence of IPF in the United States and Europe (Nalysnyk et al., 2012). Using narrow case definitions of IPF, an estimate of

14.9 to 27.9 per 100,000 people live with IPF in the United States, while the estimate increases to 42.7 to 63 per 100,000 people when using broad case definitions. Similarly, the incidence of new cases varied from 6.8 to 8.8 per 100,000 per year using narrow case definitions and 16.3 to 17.4 per 100,000 people per year using broad IPF definitions (Nalysnyk et al., 2012). In Europe, approximately 1.25 to 23.4 per 100,000 people suffer with the disease while incidence ranges from 0.22 to 7.94 per 100,000 people every year (Nalysnyk et al., 2012). In the United Kingdom, more recent data from the British Lung Foundation reported approximately 32,479 living with IPF, 7,865 new cases of IPF and 5,292 deaths in 2012 which may give a better insight on the annual numbers of incidence, prevalence and mortality (British Lung Foundation, 2018). Additionally, a more recent study by Raghu *et al.* (Raghu et al., 2016) demonstrated a similar incidence and prevalence of IPF in the United States.

Despite the discrepancies relating to disease definition, study population and design, there is clear and consistent evidence that IPF incidence and prevalence are increasing in most countries worldwide (Olson et al., 2007; Navaratnam et al., 2011; Nalysnyk et al., 2012; Raghu et al., 2016; Ley et al., 2011; Ley and Collard, 2013). IPF has the worst prognosis of all ILDs with median survival ranging between 2.5 to 3.5 years after diagnosis, with some studies reporting up to around 5 years depending on the diagnostic criteria and the stage of the disease at the time of diagnosis (Ley et al., 2011; Collard et al., 2003; Fernández Pérez et al., 2010). Interestingly, comparing statistics from the US National Cancer Institute (Vancheri et al., 2010) and IPF (Olson et al., 2007), survival rates of IPF appear to be worse compared to several types of cancer. The clinical course of IPF patients varies from slow progression to rapid deterioration and death depending on disease progression (Barlo et al., 2010). Thus, the course of IPF is unpredictable and it can be difficult to predict patterns of disease progression.

Studies have also focused on the influence of age and sex upon IPF occurrence; showing higher incidence amongst the elderly population (55-64 years old) as well as males compared to females (incidence rate ratio 1.08) (Raghu et al., 2016). IPF incidence and mortality rates have been previously

shown to be higher in men and also to increase with age (Olson et al., 2007; Navaratnam et al., 2011). The epidemiology study by Olson *et al.* has also shown that incidence appears to be higher within the white, non-Hispanic population, suggesting a role for race and ethnicity (Olson et al., 2007). Apart from sex, age and race, several additional risk factors have been linked with the development of the disease. Exposure to cigarette smoke (Baumgartner et al., 1997), metal and wood dust (Paolocci et al., 2018; Koo et al., 2017) as well as microbial infections (Molyneaux et al., 2014) are some of the environmental factors associated with IPF (Ley and Collard, 2013). Several occupations have also been linked with the development of IPF such as hairdressing, farming, agriculture and livestock handling (Baumgartner et al., 2000). Genetically susceptible individuals carrying IPF-associated gene mutations have also been shown to be more prone to developing the disease, possibly via interactions between environmental, occupational and genetic stressors (Steele and Schwartz, 2013). A number of comorbidities are also associated with IPF including lung cancer, pulmonary hypertension, emphysema, gastroesophageal reflux and cardiovascular disease (Lee et al., 2014).

1.2.2. IPF symptoms

IPF induces exertional dyspnoea, a feeling of breathlessness, which is one of the most common symptoms experienced. Dyspnoea is often accompanied with a non-productive dry cough which progressively worsens over time until it becomes clear to patients that their daily functionality is compromised. Pulmonary malfunction due to fibrosis may induce other symptoms such as inspiratory crackles, chest discomfort, finger clubbing, weakness and loss of appetite (King et al., 2001a; Oldham and Noth, 2014; Meltzer and Noble, 2008). Unfortunately, the presentation of IPF is often confused with other types of ILDs or cardiovascular diseases and may often be attributed to aging.

1.2.3. IPF diagnosis

The clinical presentation of IPF especially in the initial stages, may be unspecific and therefore a multidisciplinary team is required to rule out other types of ILDs. As such, exclusion of other ILDs with a known cause and a similar interstitial pneumonia pattern is of crucial importance and the first step towards diagnosis of IPF. Blood tests may detect the presence of serological biomarkers and autoantibodies present in other types of ILDs such as connective tissue disease-associated ILDs (Mikolasch et al., 2017). Initial investigations may also include pulmonary function tests (PFT) which are often useful for assessing the severity of the disease as well as directing further diagnostic tests. PFTs may give a better insight on the reduction of total lung capacity (TLC), forced vital capacity (FVC) and diffusing carbon monoxide (DLCO) capacity. Additional physical tests include exercise tests such as the 6-minute walk test which are used to determine abnormalities in oxygen transfer and gas exchange in the lungs (Ley et al., 2011).

However, in order to reach a consensus diagnosis of IPF, further investigations based on radiological and histological evidence are required. Medical imaging such as chest X-rays or high resolution computed tomography (HRCT) enable detection of the presence of an usual interstitial pattern, a key histological characteristic of IPF lungs (Mikolasch et al., 2017). HRCT has now become a standard test as it can provide invaluable information and a more detailed evaluation of the extent of fibrosis compared to x-rays which are more antiquated and may lack diagnostic reliability in this context. Using HRCT has improved the overall diagnostic accuracy of IPF and reduced the necessity for more invasive tests such as surgical lung biopsies, a relatively higher risk procedure that not all patients may be fit enough to partake in. Typical HRCT shows features of interstitial pneumonia including honeycombing with sub-pleural and basal abnormalities, reticular opacities and often traction bronchiectasis. Additionally, bronchoalveolar lavage (BAL) may be undertaken to examine total cell counts, infection or the presence of malignant cells in order to exclude other causes of ILDs. In some cases, a surgical lung biopsy may be recommended to confirm diagnosis in patients

where HRCT fails to detect the presence of a definite IPF pattern. Tissue obtained from biopsies may provide further histological evidence of honeycombing, fibrosis and scarring in the pulmonary parenchyma (Meltzer and Noble, 2008; Oldham and Noth, 2014; Sgalla et al., 2016).

Updated guidelines for the diagnosis and management of IPF have been published in 2011 in a joint effort from the ATS, ERS, the Japanese Respiratory and Latin-American Thoracic societies (Raghu et al., 2011).

1.2.4. The pathogenesis of IPF

Under physiological conditions, fibrogenesis is initiated in response to tissue injury and forms part of the wound repair process involved in the restoration of homeostasis. Fibrogenesis is commonly initiated by epithelial injury, leading to activation of the coagulation and inflammation cascades. This in turn results in the activation, recruitment and proliferation of fibroblasts that are responsible for the release of ECM components. In the final remodelling stage, the wound area is resolved and normal tissue structure and structural integrity is restored (Strieter, 2008).

During the fibrosis associated with IPF, any stage of the wound repair process may be dysregulated **(Figure 1)**, resulting in the irreversible accumulation of scar tissue. These fibrotic areas are characterised by an overproduction of ECM components, predominantly collagen as well as other fibrotic proteins, which dramatically remodels the lung architecture and leads to excessive scarring (Wynn, 2011). The secretion of several pro-fibrotic cytokines and growth factors are thought to be crucial mediators of fibrosis. These drive the migration, proliferation and activation of mesenchymal cells which ultimately results in the differentiation of fibroblasts into α-smooth muscle actin (α-SMA)-expressing myofibroblasts and the development of fibrotic foci, a dense collection of myofibroblasts and scar tissue (Hinz et al., 2007). The pleiotropic growth factor TGF-β1 (transforming growth factor β1) is regarded as a key player of fibrosis (Desmoulière et al., 1993) along with other mediators such as platelet-derived growth factor (PDGF) (Antoniades et al., 1990), interleukin

1β (IL-1β) (Zhang et al., 1993) and several chemokines (Sahin and Wasmuth, 2013) and cytokines (Borthwick et al., 2013).

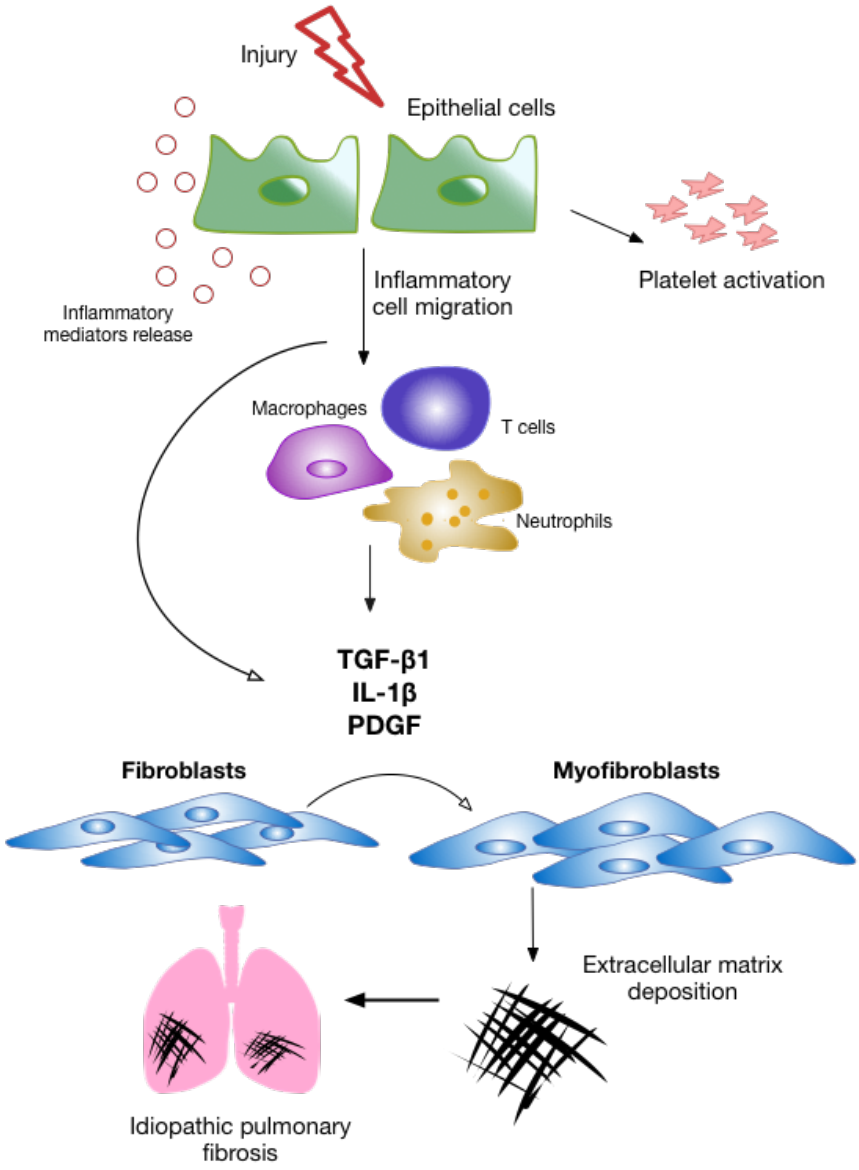

Figure 1. Overview of the most significant wound healing stages leading to the development of IPF. Epithelial cell injury leads to the secretion of inflammatory mediators and triggers platelet activation which results in enhanced vessel permeability for the recruitment of leukocytes. These inflammatory cells release pro-fibrotic cytokines such as TGF-β1 that mediate the activation and recruitment of fibroblasts as well as their differentiation into myofibroblasts and the subsequent release of ECM components to promote wound healing. In IPF, an aberrant wound repair response leads to the irreversible formation of excessive scar tissue in the lungs.

At the present time, the drivers of the aberrant wound healing process leading to fibrosis are unknown. However, certain cells types such as alveolar epithelial cells and fibroblasts, as well as a dysregulated coagulation and inflammatory response have been implicated in disease initiation and progression (King et al., 2011; Sgalla et al., 2018; Kendall and Feghali-Bostwick, 2014; Martinez et al., 2017).

1.2.4.1. Alveolar epithelial injury

Prior to the activation of fibroblasts, it is believed that type I AECs maybe subjected to repetitive injury, which causes damage to the delicate epithelium structure. Type I AECs line more than 90% of the alveolar surface and damage promotes the activation and proliferation of the surfactant-producing type II AECs (Shannon and Hyatt, 2004). This results in hyperplasia of the type II AECs in order to cover the exposed alveolar surface, as well as the activation of local coagulation pathways and the initiation of a provisional matrix also known as a wound clot (Chambers, 2008a).

During the normal healing process, the lung tissue will eventually regain its original structure and function as the provisional matrix gradually dissipates. In the case of injury of the type I AECs, the hyperplastic type II AECs are thought to undergo regulated apoptosis and trans-differentiation into type I AECs in order to re-establish a fully functional alveolar epithelium (Selman and Pardo, 2006).

However, if the epithelial basement membrane remains disturbed following extensive damage, the alveoli can collapse and type II AECs fail to undergo re-epithelisation. An aberrant wound repair response may then be initiated during which the epithelial cells, predominantly type II AECs, are thought to release several pro-fibrotic cytokines, growth factors and other chemokines at the site of injury (Allen and Spiteri, 2002). The epithelial injury is then thought to ultimately promote the activation and proliferation of fibroblasts and myofibroblasts and the formation of a stiffened ECM in IPF (Camelo et al., 2014; Kasper and Barth, 2017).

1.2.4.2. Coagulation

In the initial stages of wound repair, circulating platelets are activated via the coagulation cascade to promote platelet aggregation and the formation of cross-linked fibrin strands at the site of injury (Crooks et al., 2014). The coagulation cascade is tightly regulated by endogenous anti-coagulants and the action of thrombin (Chambers, 2008a). Fibrin deposition was shown to be present in the lungs of IPF patients whilst other reports have shown elevated expression of Tissue Factor (TF) in type II AECs (Imokawa et al., 1997) and BAL fluid (Kotani et al., 1995).

Upon tissue injury, TF forms the TF-activated factor VIIa (FVIIa) to initiate the coagulation cascade. The coagulation cascade is a complex process, but TF-FVIIa complex ultimately triggers the activation of factor X (FX) to factor Xa (FXa); FXa in association with activated factor V (FVa) leads to the conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin resulting in the subsequent formation of a wound clot. Interestingly, a study by Scotton *et al*. (Scotton et al., 2009) demonstrated elevated expression of FX and the proteinase FXa in the bleomycin-induced fibrotic lungs. FXa was shown to contribute to driving the fibrotic response by inducing myofibroblast differentiation via a TGF-β-dependent mechanism, involving the thrombin receptor and proteinase-activated receptor-1 (PAR1).

Fibrin may be broken during the process of fibrinolysis via the activation of plasmin. Plasmin is converted from plasminogen by the action of proteinases, urokinase-type plasminogen activator and tissue-type plasminogen activator (Chambers, 2008b). Studies have shown that inhibitors of this pathway such as plasminogen activator inhibitor 1 (PAI-1) are elevated in IPF patients (Kotani et al., 1995) and bleomycin-induced fibrosis (Eitzman et al., 1996) indicating a pro-coagulant activity in IPF lungs (Crooks and Hart, 2015). Based upon these observations, the process of coagulation has been targeted for potential therapeutics, although anti-coagulants are still not recommended for IPF patients (Raghu et al., 2011).

1.2.4.3. Inflammation

One of the initial concepts relating to IPF pathogenesis was that the disease was initiated and driven by chronic inflammation. However, the role of inflammation was questioned when anti-inflammatory and immunosuppressant therapies failed to improve lung function and survival of IPF patients. A study of 330 IPF patients showed no effect on progression-free survival when treated with the immune-regulatory cytokine Interferon gamma-1β (IFN-γ-1β) (Raghu et al., 2004). Additionally, the INSPIRE randomised double-blinded placebo-controlled trial of IFN-γ-1β did not show any benefit compared to the placebo and was terminated (King et al., 2009). The IFIGENIA trial, a double-blind, randomised, placebo-controlled study assessed the efficacy of N-Acetylcysteine (NAC) added to prednisone and azathioprine (Demedts et al., 2005), however the survival benefit of IPF patients was questioned. The follow-on PANTHER trial was designed to address some of the issues of the IFIGENIA trial and evaluated the response in IPF patients of this triple therapy, but was terminated prematurely when it showed significantly increased mortality (Idiopathic Pulmonary Fibrosis Clinical Research Network et al., 2012). The study continued as a two-group study (NAC vs. placebo) but demonstrated no benefit to IPF patients (Martinez, 2014).

Whilst the role of inflammation in the initiation and progression of the disease remains unclear, there is plenty of evidence to indicate that IPF is associated with inflammation and changes in the innate and adaptive immune response (Wick et al., 2013). Studies have shown that expression of inflammatory chemokine (C-C motif) ligands (CCL) such as CCL2 (Antoniades et al., 1992), CCL11 (Huaux et al., 2005) and CCL8 (Lee et al., 2017) are elevated in fibrotic lungs. The secretion of the pro-inflammatory cytokine IL-1β has been linked to the progression and development of fibrosis by enhancing the expression of the inflammatory mediators interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), disrupting alveolar architecture and by increasing pulmonary fibroblasts and collagen deposition (Kolb et al., 2001). Release of the pro-fibrotic cytokines TGF-β1 and PDGF may also be stimulated by IL-1β in BAL

fluid (Kolb et al., 2001). IL-1β was also shown to increase the infiltration of neutrophils and macrophages to the lungs (Kolb et al., 2001; Lappalainen et al., 2005) and elevate the expression of matrix metalloproteinases (MMPs) MMP-9/12 and chemokine (C-X-C motif) ligands (CXCL) CXCL1/2 (Lappalainen et al., 2005).

Inflammatory cells found in the lungs of IPF patients were shown to produce elevated levels of reactive oxygen species (ROS) which are thought to contribute to tissue damage in IPF (Cantin et al., 1987; Strausz et al., 1990). Mitochondria-derived ROS may also drive pro-inflammatory cytokine production including IL-1β expression (Naik and Dixit, 2011). ROS production was shown to be regulated by TGF-β1 and to further mediate downstream cellular events such as IL-6 expression (Junn et al., 2000) and activation of PAI-1 (Vayalil et al., 2007), an important regulator of ECM degradation. As such, in this environment where fibrotic and inflammatory mediators work together, an acute lung injury may readily escalate into a chronic fibrotic response; thus, controlling the acute inflammatory activity may prove beneficial in eliminating the downstream effects of a chronic progressive fibrotic state.

The adaptive immune response has also been linked to the development of IPF. Interestingly, the pro-inflammatory cytokine interleukin 17A (IL-17A) which is expressed by $CD4^+$ T-helper (T_H-17) cells, has been linked with enhanced neutrophil recruitment, also known as neutrophilia, as well as TGF-dependent and IL-1β-driven fibrosis (Wilson et al., 2010). Notably, a study by Kinder et *al.* demonstrated that elevated levels of neutrophils in BAL fluid was shown to be a prognostic predictor of early mortality in IPF patients (Kinder et al., 2008). Additionally, T_H-1 effector T cells are thought to exert anti-fibrotic activities through the production of IFN-γ, which was shown to attenuate fibrosis (Baroni et al., 1996) by inhibiting TGF-β-induced phosphorylation of Smad3 (Ulloa et al., 1999). T_H-2 effector T cells are thought to promote fibrosis via the production of pro-inflammatory cytokines such as interleukin 13 (IL-13) which stimulates collagen deposition in fibroblasts (Chiaramonte et al., 1999).

In this environment, activated platelets, damaged epithelial cells and recruited inflammatory cells release more pro-fibrotic growth factors such TGF-β1 that drive the fibrotic response. TGF-β1 is considered the driving force of fibrosis as it has multiple properties including activation of fibroblasts, epithelial cell differentiation via epithelial-mesenchymal transition (EMT) and even stimulation of the expression of pro-inflammatory cytokines such as IL-1β to further enhance the fibrotic response (Wynn and Ramalingam, 2012).

1.2.4.4. Lung fibroblasts

Lung fibroblasts are also activated in response to epithelial injury and thought to be important in the development of pulmonary fibrosis. Fibroblasts are activated at the site of injury following the release of pro-fibrotic mediators such as TGF-β1 (Border and Noble, 1994). Under these pathologic conditions, fibroblasts may differentiate into myofibroblasts, a cell type that is associated with the fibrotic lung and predominantly located within fibrotic foci (Kuhn and McDonald, 1991; Zhang et al., 1994). Myofibroblasts share features with smooth muscle cells as they express α-SMA stress fibres and present a more contractile and pro-fibrotic potential phenotype (Hinz et al., 2001). Specifically, they are believed to secrete excessive amounts of ECM components such as collagen, hyaluronic acid, elastin, proteoglycans and other proteins that are deposited in the ECM (King et al., 2011; Pardo and Selman, 2016).

Myofibroblasts that accumulate in the lungs of IPF patients are a central feature of the disease and are thought to originate from at least three different sources. The most prominent and straightforward source is the proliferation and differentiation of resident fibroblasts following the release of pro-fibrotic mediators such as TGF-β1 (Desmoulière et al., 1993). Pro-fibrotic mediators also promote the influx and migration of circulating fibrocytes to the wound site (Abe et al., 2001). Fibrocytes are bone marrow mesenchymal cells which are present in the blood and tissues in a relatively inactive state until chemotactic stimuli such as CXCL12 promote their migration to the lungs where they can contribute to the development of pulmonary fibrosis (Phillips

et al., 2004) and give rise to collagen-producing fibroblasts (Hashimoto et al., 2004). Finally, it has been proposed that alveolar epithelial cells may acquire mesenchymal phenotypes via the process of EMT, during which they lose their epithelial cell markers and transform into fibroblast-like cells as demonstrated by Kim *et al*. (Kim et al., 2006). EMT-mediated formation of fibroblasts was also shown to be regulated by exposure to TGF-β1 (Willis et al., 2005), indicating the possibility of epithelial cells serving as a source of myofibroblasts. The contribution of fibrocytes, EMT and other cell types such as pericytes (Hung et al., 2013) to the fibroblast population in the progression of IPF is still unclear.

1.2.4.5. The fibrotic response and ECM remodelling phase

The aberrant wound healing response in IPF involves several underling mechanisms which are thought to drive the disease. However, the most well-established and studied concept is the role of TGF-β1 in the development of fibrosis and its effect on fibroblasts and the ECM which play a major role in the fibrotic cascade (Roberts et al., 1986). TGF-β1 activates a complex network of intracellular pathways and exhibits several properties that are thought to promote fibrosis such as EMT (Willis et al., 2005), apoptosis (Kim et al., 2006), as well as recruitment and proliferation of fibroblasts via PDGF expression which can also release more TGF-β1 (Scotton and Chambers, 2007). TGF-β1 is secreted by most cells, however AECs are the main source of TGF-β1 during the initial stages of wound repair. TGF-β1 may also regulate the expression of several pro-inflammatory and pro-fibrotic mediators and work with them synergistically to further enhance the fibrotic response (Fernandez and Eickelberg, 2012).

Most importantly, in this fibrotic environment, both fibroblasts and myofibroblasts secrete increased amounts of ECM components to synthesise and maintain the ECM. The ECM is a complex and versatile network of cross-linked and fibrous proteins that form a protective structure under healthy conditions; however, the excessive deposition of the matrix in IPF is thought to exert powerful effects on cell functions via ECM-cell interactions (Clarke et

al., 2013). As such, secreted TGF-β1 was shown to be a potent inducer of ECM production (Broekelmann et al., 1991), whereas the mechanical stress of ECM and contractile myofibroblasts further stimulate the activation of TGF-β1 (Wipff et al., 2007). Fibroblasts are also capable of matrix degradation by producing MMPs and the tissue inhibitors of metalloproteinases (TIMPs) **(Figure 2)**. However, the highly complex nature of ECM biology is reflected by the diverse roles of MMPs and TIMPs in fibrosis where they demonstrate both pro- and anti-fibrotic activities during the tissue remodelling phase (Giannandrea and Parks, 2014). Interestingly, the expression of several ECM-degrading enzymes were shown to be elevated in the IPF lungs, however the excessive deposition of ECM components results in the accumulation and the development of a stiff matrix and ultimately lung fibrosis (Pardo et al., 2008; Kulkarni et al., 2016).

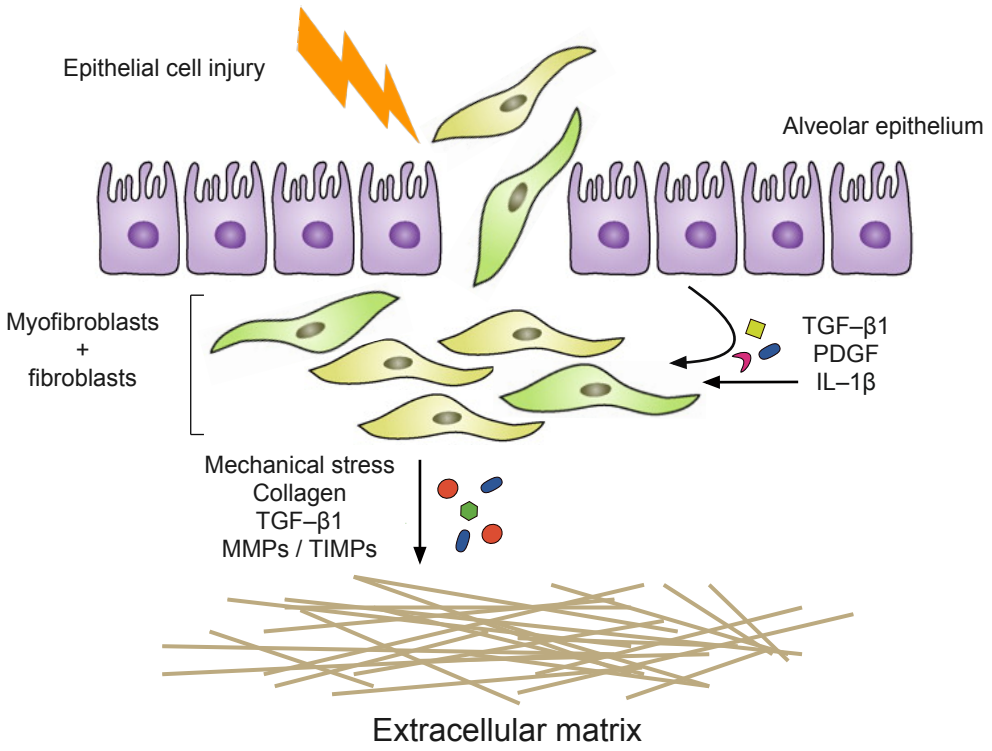

Figure 2. Pulmonary fibroblasts interact with the extracellular matrix to enhance the fibrotic response. During the fibrotic response, the alveolar epithelial cells undergo apoptosis due to injury which results in the infiltration of fibroblasts and myofibroblasts into the alveolar space. The dysregulation of fibroblasts is thought to be a critical player in the development of IPF where they excessively synthesize and release extracellular matrix components. The

matrix is maintained by the presence of pro-fibrotic mediators released by the fibroblasts and other cells. The interactions of the fibroblasts and the ECM further enhance the fibrotic response in a positive-feedback loop.

1.2.5. TGF-β1 signalling

TGF-β1 is one of the three isoforms of the TGF-β superfamily of growth factors which exerts its fibrotic effects by binding to the transmembrane TGF-β1 (TβRI) and TGF-β2 (TβRII) receptors to initiate its signalling cascade. Briefly, signalling begins when secreted TGF-β1 binds to latent TGF binding proteins (LTBPs) and latency associated proteins (LAP) to form a large latent complex (LLC) which acts as a reservoir of inactive latent TGF-β1 in the ECM (Annes et al., 2003). TGF-β1 may be 'activated' and bind to its receptors only when it is liberated from the LLC complex by protease-mediated cleavage (Mu et al., 2002) or by integrin-mediated interactions (Xu et al., 2009). Binding of TGF-β1 to the TβRs leads to the formation of ligand-receptor heterotetrametric complexes and the phosphorylation of the cytoplasmic TβRI GS domain which initiates intracellular signalling via its interaction and phosphorylation of Smad2/Smad3 (R-Smads) (Shi and Massagué, 2003). The phosphorylation of Smad2/Smad3 leads to their partnering with the signalling transducer Smad4 and ultimately their translocation to the nucleus where they form transcriptional complexes to regulate gene expression (Zi et al., 2012). The Smad family of transcriptional activators form the canonical pathway of TGF-β1 signalling, however several non-canonical TGF-β1 signalling pathways that complement Smad action have been identified to play a role in fibrosis **(Figure 3)**. TGF-β1 has been shown to activate the Erk / mitogen-activated protein kinase (MAPK) pathway to mediate myofibroblast differentiation (Caraci et al., 2008) as well as collagen and connective tissue growth factor expression (Pannu et al., 2007). Additionally, TGF-β1-mediated p38 MAPK signalling has been shown to play a role in ECM production (Furukawa et al., 2003). The c-Jun N-terminal kinase (JNK) / MAPK signalling may also be activated by TGF-β1 to induce fibronectin synthesis (Hocevar et al., 1999). TGF-β may also regulate the fibrotic response via the activation of the phosphatidylinositol-3-kinase (PI3K) / Akt (Wilkes et al., 2005; Conte et al.,

2011; Mercer et al., 2016) and the Rho GPTase (Masszi et al., 2003) signalling pathways.

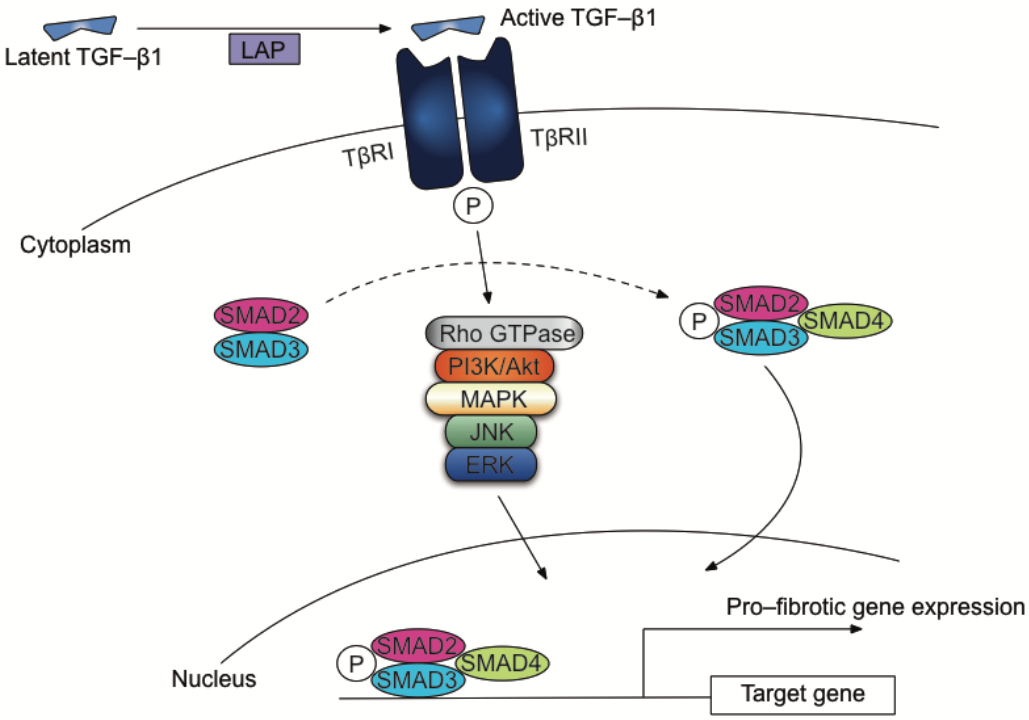

Figure 3. Activation of the Smad signalling pathway by TGF-β1 in pulmonary fibrosis. Latent TGF-β1 interacts with latency associated proteins (LAP) to form the large latent complex (LLC) which acts as a reservoir before the release of active TGF-β1. Upon activation of the TβR receptors by TGF-β1, the Smad intracellular signalling cascade is initiated (canonical pathway) which promotes the transcription of pro-fibrotic genes. Other signalling pathways (non-canonical pathways) such as MAPK and PI3K/Akt may also be activated by TGF-β1. P = phosphorylation.

1.2.6. PDGF signalling

The PDGF family of growth factors has been known to regulate cell growth and proliferation as potent mitogens of mesenchymal cells including myofibroblasts that play a crucial role in the development of fibrotic diseases (Bonner, 2004; Noskovičová et al., 2015). PDGF is primarily produced by platelets, vascular endothelial cells, alveolar macrophages and epithelial cells in human lungs. The PDGF family consists of the four polypeptide chains

PDGF-A, PDGF-B, PDGF-C and PDGF-D which form the biologically active homodimers including PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD as well the heterodimer PDGF-AB via a disulphide-bond linkage. PDGF-A and PDGF-B isoforms (-AA, -BB, -AB) are known to play important roles in fibrosis by binding to the PDGFR-α and -β receptors (Donovan et al., 2013), however much less is known about the activity of PDGF-C and PDGF-D isoforms (Beyer and Distler, 2013; Ying et al., 2017). Ligand-receptor binding leads to receptor dimerization and auto-phosphorylation which results in the phosphorylation of intracellular tyrosine kinase residues and the initiation of downstream signalling. PDGF-mediated signalling has been shown to activate the Ras-MAPK, phospholipase Cγ (PLCγ) signalling pathways to exert its mitotic effects (Andrae et al., 2008; Trojanowska, 2008) as well as the FAK (focal adhesion kinase) / PI3K / Akt (Reif et al., 2003) and signal transducer and activator of transcription 3 (STAT3) (Vij et al., 2008) signalling pathways to mediate the activity of fibroblasts **(Figure 4)**.

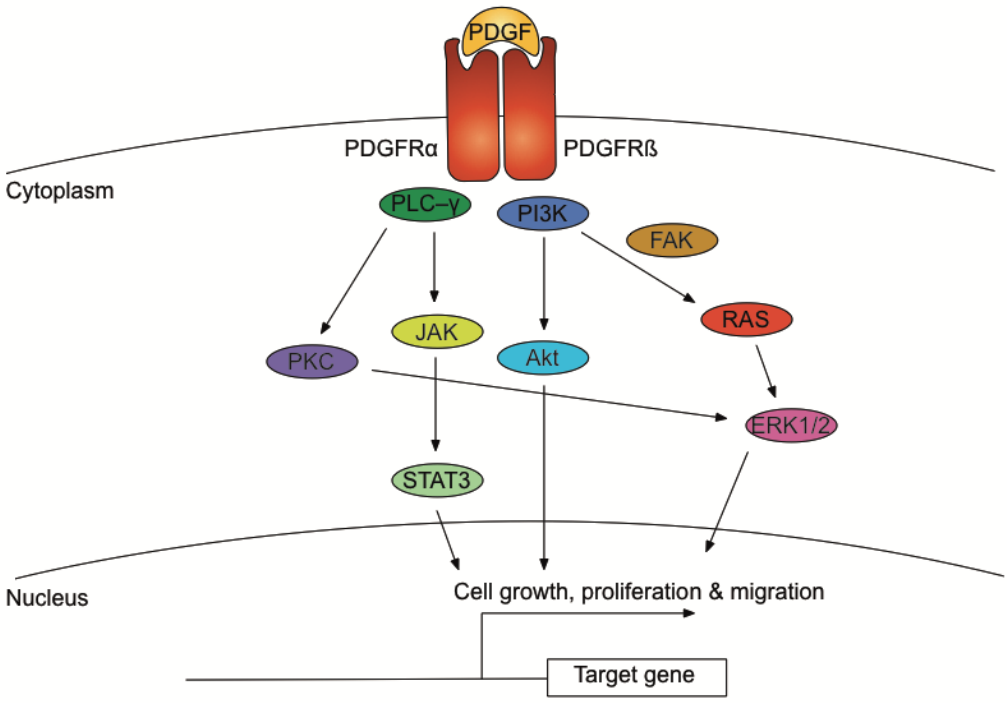

Figure 4. PDGF-A and PDGF-B isoforms are key players in PDGF signalling in pulmonary fibrosis. Activation of the PDGF receptors by PDGF isoforms leads to the transcription of genes involved in cell growth, proliferation and migration. Multiple intracellular signalling pathways have

been shown to be activated in pulmonary fibrosis by PDGF molecules such as JAK (Janus kinase) /STAT and PI3K/Akt pathways.

1.2.7. IL-1β signalling

IL-1β belongs to the IL-1 family of 11 cytokines and is primarily produced by monocytes, macrophages and dendritic cells, although B lymphocytes and Natural Killer (NK) cells are also known sources (Dinarello, 2009; Garlanda et al., 2013). IL-1β is a pro-inflammatory cytokine that is not normally expressed in healthy tissue, however it may be induced by the activation of the transcription factor, nuclear factor-κB (NF-κB) **(Figure 5)**. The inactivate IL-1β pre-cursor is cleaved by caspase-1 to become active in response to inflammatory signals initiated by pathogens or other inflammatory cytokines. However, prior to cleavage of the IL-1β pre-cursor by caspase-1, a complex of intracellular proteins known as the inflammasome activate pro-caspase-1 to the active caspase-1 (Latz et al., 2013). In most cells, caspase-1 is maintained in the inactive form in order to keep IL-1β activation highly regulated, however caspase-1 appears to be constitutively activated in human monocytes (Netea et al., 2009). An influx of calcium in the cell initiates the secretion of mature IL-1β which binds to the IL-1 receptors (IL-1R). Binding to the IL-1R1 initiates the downstream signalling cascade, however IL-1R accessory protein and IL-1R2 appear to act as co-receptors which interact with IL-1R1 to regulate its activity (Sims and Smith, 2010). The signalling cascade is initiated upon engagement of MyD88 (Myeloid differentiation factor 88) adapter protein which is required for the activation of IRAK1 and IRAK2 kinases through IRAK4. The IRAKs interact with E3 ubiquitin protein ligases such as TRAF6 (TNF receptor-associated factor 6) which ultimately leads to the recruitment of TAB2 and TAB3 which form the TAK1 (Transforming growth factor beta-activated kinase) kinase complex. TAK1 activates the IκB kinase (IKK) complex, also known as IKKγ, to induce activation of the NF-κB transcription factors (Cui et al., 2014; Gañán-Gómez et al., 2015). TAK1 was also shown to activate the MAPK signalling pathways p38α (Mitogen-activated protein kinase 14) and JNK (Newton and Dixit, 2012).

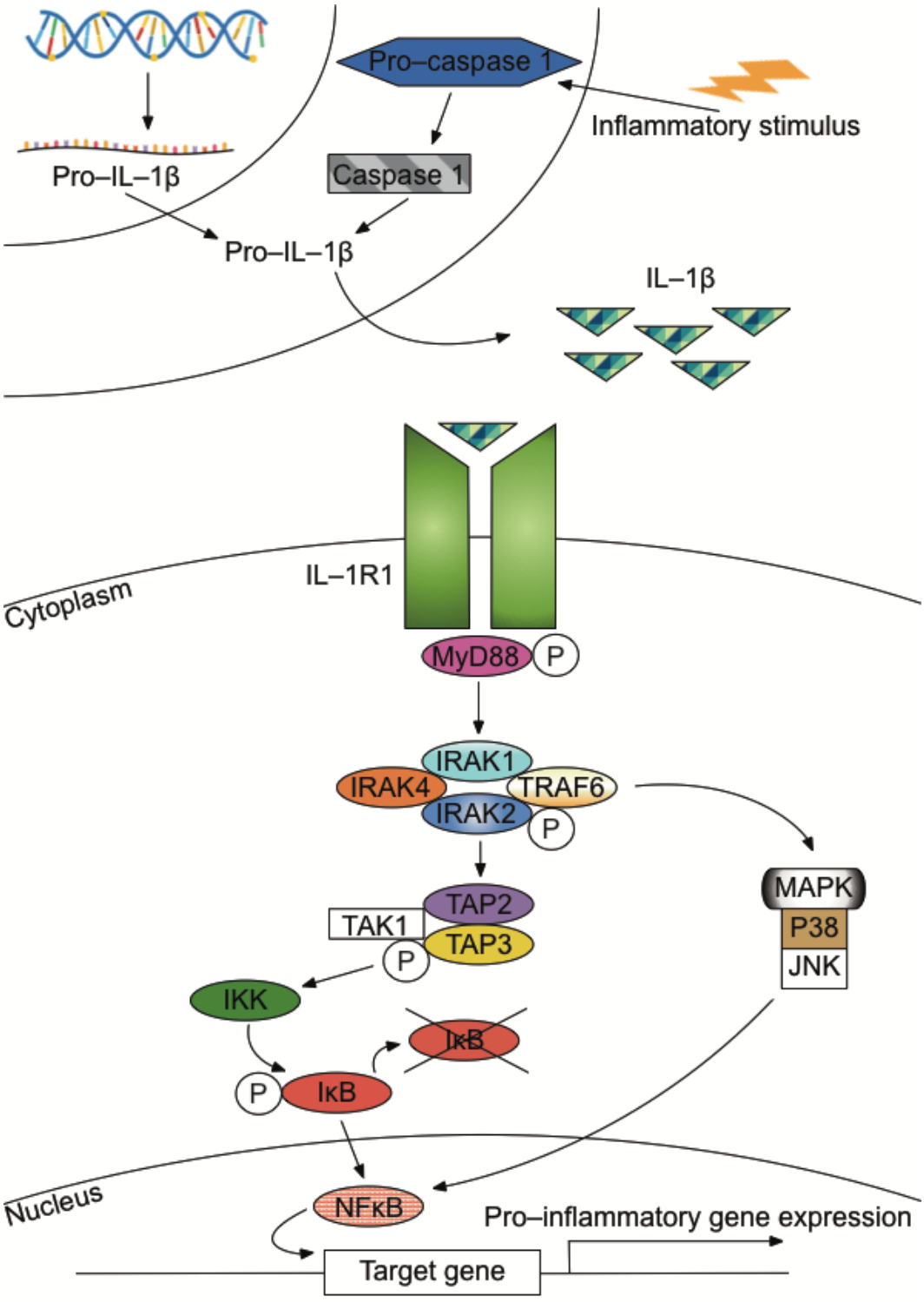

Figure 5. Activation of the NF-κB signalling pathway by IL-1β. Upon activation of the IL-1 receptors by IL-1β, the recruitment of adaptor proteins such as MyD88 initiate the activation of intracellular signalling pathways to activate transcription factors such as NF-κB. The activation of such signalling cascades ultimately results in the transcription of pro-inflammatory genes that have been shown to mediate cellular events in pulmonary fibrosis. P = phosphorylation.

1.2.8. Genetic studies in IPF

IPF is a heterogeneous disease that is characterised by complex genetic and environmental interactions that contribute to the development of the disease; hence it is now thought that genetically susceptible individuals exposed to environmental stressor stimuli have an increased risk of developing the disease. Both rare and common genetic variants have been associated with sporadic and familial pulmonary fibrosis (Kaur et al., 2017).

Genetic studies have linked IPF in adults to rare genetic variants in surfactant protein-related genes. Mutations have been found in the genes such as surfactant protein C (SFTPC) and A2 (SFTPA2) (Coghlan et al., 2014) which may result in alveolar epithelial cell injury following a disruption in their synthesis. Specifically, it appears that the SFTPC mutation causes defects in protein folding within the endoplasmic reticulum (ER) of type II AECs, which was associated with IPF progression (Lawson et al., 2008). Similarly, the SFTPA2 mutation was shown to enhance ER stress and has been associated with the development of pulmonary fibrosis (Maitra et al., 2010; Spagnolo and Cottin, 2017).

Rare variants in several genes regulating telomere biology have also been identified in IPF patients, particularly mutations affecting telomerase and telomerase-associated proteins such as TERT (Armanios et al., 2007; Coghlan et al., 2014) and TERC (Tsakiri et al., 2007). Telomeres are found at the end of chromosomes and protect them from DNA damage during the replication process. TERT and TERC encode telomerase genes which restore telomere length and mutations lead to increased telomere shortening. Interestingly, IPF has been associated with telomere shortening (Alder et al., 2008), although the exact mechanisms that links this to the fibrotic response are still unclear.

The single-nucleotide polymorphism rs35705950, located in the putative promoter region of the MUC5B gene was also found to play a role in predisposing patients to familial and sporadic forms of IPF by causing increased MUC5B expression (Seibold et al., 2011). The MUC5B gene encodes for mucin 5B, a gel-forming protein, expressed by epithelial cells.

However, upregulated MUC5B expression is also observed in IPF patients in the absence of rs35705950 indicating alternative mechanisms that may increase MUC5B expression which are currently being investigated (Steele and Schwartz, 2013).

1.2.9. Gene expression studies in IPF

Transcriptional changes in the lungs of IPF patients have also contributed to our understanding of the disease and its underlying molecular mechanisms. In particular, microarray and sequencing based approaches have identified an association between IPF and ECM formation, smooth muscle markers, growth factors, chemokines and immunoglobulins (Vukmirovic and Kaminski, 2018).

Several gene expression profiling studies have demonstrated widespread changes in the profile of mRNA (messenger RNA) expression in lung biopsies. A study by Nance *et al*. identified 873 differentially expressed genes in IPF lung biopsies compared to controls using RNA-sequencing (RNA-seq). Interestingly, 675 of these genes displayed alternative splicing events including those coding for periostin (POSTN) and collagen (COL6A3) (Nance et al., 2014). Microarrays showed differential expression of 2940 genes in IPF lung tissue compared to controls, including genes encoding for collagens, proteinases, cytokines and growth factors (DePianto et al., 2015). The gene expression profile of control and IPF lung biopsies was also assessed using microarrays by Bridges *et al*. (Bridges et al., 2009). The Twist1 gene was the most consistently up-regulated in IPF lungs and was found to have a protective role against apoptosis. Gene expression was also assessed in the lungs of IPF patients to identify mechanisms of acute exacerbations. This study identified 579 differentially expressed genes including CCNA2 and α-defensins which were amongst the most up-regulated genes (Konishi et al., 2009). In another study, comparison of lung biopsies from relatively stable and progressive IPF patients, demonstrated differential expression of 243 transcripts including CCL2 and SFTPA1 (Boon et al., 2009).

As well as biopsies, gene expression in isolated human lung fibroblasts has

also been employed to examine and identify novel IPF-related genes and pathways. A recent study by Lee *et al.* identified CCL8 expression to be elevated in IPF lung fibroblasts using microarrays (Lee et al., 2017). Another report by Plantier *et al*. used publicly available microarray data to analyse and compare the expression of genes in cultured control and IPF fibroblasts (Plantier et al., 2016). Notably, two of the most significantly expressed factors were the connective tissue growth factor (CTGF) and serum response factor (SRF) which were shown to be overexpressed in IPF fibroblasts. Gene expression of lung fibroblasts was also assessed by microarrays following 4 hour treatment with TGF-β1 (Renzoni et al., 2004). The expression of 129 transcripts was shown to be driven by TGF-β1 stimulation including SMAD specific E3 ubiquitin protein ligase 2 (SMURF2), bone morphogenetic protein 4, and angiotensin II receptor type 1 (AGTR1).

In an attempt to identify circulating biomarkers of IPF, transcriptome analysis has also been undertaken on blood serum and plasma. A study by Yang *et al.* used microarrays to evaluate circulating genes in IPF patients based on disease severity. The study identified 1428 differentially expressed transcripts in the peripheral blood of mild IPF and 2790 differentially expressed transcripts in severe IPF compared to control patients. The genes encoding for MMP9 and IL-1R2 were found to be upregulated in both mild and severe IPF patients (Yang et al., 2012). Elevated levels of systemic MMP3 and CXCL13 were also identified in the blood of IPF patients using microarrays (DePianto et al., 2015).

1.2.10. Current pharmacological strategies for the treatment of IPF

There are currently two drugs that have been approved for the treatment of IPF, nintedanib and pirfenidone, both of which slow the decline of lung function associated with the disease.

Nintedanib, also known as BIBF1120, was originally developed as an anti-cancer agent and is thought to be a non-specific tyrosine kinase inhibitor. A study by Hilberg *et al.* showed intracellular inhibition of the receptors for vascular endothelial growth factor (VEGFR), fibroblast growth factor (FGFR) and platelet-derived growth factor (PDGFR) by nintedanib (Hilberg et al., 2008). Evidence that nintedanib has anti-fibrotic activities came from reports showing attenuated fibrosis in the bleomycin-induced model of lung fibrosis and inhibition of TGF-β-induced fibroblast to myofibroblast differentiation *in vitro* (Chaudhary et al., 2007; Wollin et al., 2014). It was also shown to inhibit collagen deposition induced by TGF-β in human lung fibroblasts *in vitro* (Hostettler et al., 2014). Nintedanib might also have anti-inflammatory activity following the observation that it reduced IL-1β production and lymphocyte counts in bronchoalveolar lavage fluid (BALF) obtained from human fibrotic lungs (Wollin et al., 2014). In 2014 nintedanib was approved by the US Food and Drug Administration (FDA) for oral use in IPF patients after the completion of the INPULSIS-1 and IMPULSIS-2 trials (Richeldi et al., 2014). The IMPULSIS trials were two 52-week parallel phase III randomised, double-blind trials to evaluate its safety and efficacy in a total of 1066 patients. Overall, nintedanib was shown to reduce the decline of FVC and was considered safe, with no significant increase in mortality rates compared to the placebo group. Mild to moderate diarrhoea was reported to be the most frequent adverse effect experienced by patients along with other events such as nausea and vomiting.

Pirfenidone is thought to be an anti-oxidant and anti-inflammatory compound which was first shown to have anti-fibrotic activities in the bleomycin model of lung fibrosis (Spagnolo et al., 2015b). It was suggested to exert an anti-fibrotic effect by targeting and inhibiting TGF-β expression (Iyer et al., 1999). Pirfenidone was administered to terminally ill IPF patients for the first time in a clinical setting as part of a small open-label phase II study by Raghu *et al.* which showed promising results with satisfactory tolerability and relatively minor adverse events (Raghu et al., 1999). Pirfenidone has been shown to reduce levels of monocyte chemoattractant protein (MCP) -1, TGF-β, IFN-γ, FGF, IL-6, IL-1β and other cytokines in the bleomycin-fibrosis model (Oku et

al., 2008). Human lung fibroblast proliferation and TGF-β-induced differentiation is also inhibited by pirfenidone *in vitro*. Phosphorylation of Smad3, p38, and Akt, which are key molecules in the TGF pathway, were also decreased by pirfenidone (Conte et al., 2014), although its exact mechanism of action is still unclear. Pirfenidone was approved as the first anti-fibrotic therapy for IPF following the demonstration of improved mortality and reduced disease progression during the CAPACITY (Noble et al., 2011) and ASCEND trials (King et al., 2014). The CAPACITY trials (studies 004 and 006) evaluated the oral administration of pirfenidone for at least 72 weeks. Paradoxically, although one trial (004) showed an overall reduction in the decline of FVC, this was not observed in the other trial (006) (Noble et al., 2011). This led to the ASCEND trial which evaluated the administration of pirfenidone in 555 patients over 52 weeks. In this trial, pirfenidone was shown to slow the decline in lung function, improve exercise tolerance and to be generally safe and well tolerated with an acceptable adverse event profile (King et al., 2014).

1.3. Non-coding RNAs

Initial data from the Human Genome Project indicated that there were approximately 30,000 to 40,000 protein coding genes in the human genome (Lander et al., 2001). However, this estimate was significantly reduced to 20,000 to 25,000 in the final draft published three years later (International Human Genome Sequencing Consortium, 2004). In terms of the total length of the human genome (approximately 3 billion), this meant that just 1-2% codes for the exonic regions of proteins. Although the function of the remaining DNA is currently an area of investigation, we know that much of this is transcribed into RNA which is not translated and is therefore classified as 'non-coding RNA' (ncRNA) (Kaikkonen et al., 2011; Morris et al., 2014). Despite being initially considered as 'junk', ncRNAs are now known to have multiple biological functions and play a significant role in health and disease (Esteller et al., 2011; Kopp and Mendell, 2018).

The term ncRNA includes all RNA molecules that do not encode for proteins, a very big proportion of these are the well-known 'housekeeping' RNAs including transfer RNAs and ribosomal RNAs, which play a critical role in protein biosynthesis. In addition, there are the small nucleolar RNA (snoRNA), which regulate the transcriptional modification of other RNA species and the small nuclear RNAs (snRNA), that form an important component of the spliceosome complex, which removes introns from mRNA during transcription. In total, these 'housekeeping RNAs" represent around 85-90% of the total RNA whilst the mRNAs account for 5-8%. The remaining ncRNAs are speculated to have regulatory roles in various cellular functions and are divided in two classes, the short (< 200 nucleotides) which are exemplified by the microRNA (miRNA) and long (> 200 nucleotides) non-coding RNAs (lncRNAs) (Kaikkonen et al., 2011). miRNAs are thought to predominantly regulate gene expression at the translational level and lncRNAs at the transcriptional level **(Figure 6)**.

Regulatory ncRNAs are now believed to regulate multiple biological responses whilst their aberrant expression has been linked to pathologic conditions. This introduction will provide an overview of lncRNAs and their role in the development of IPF.

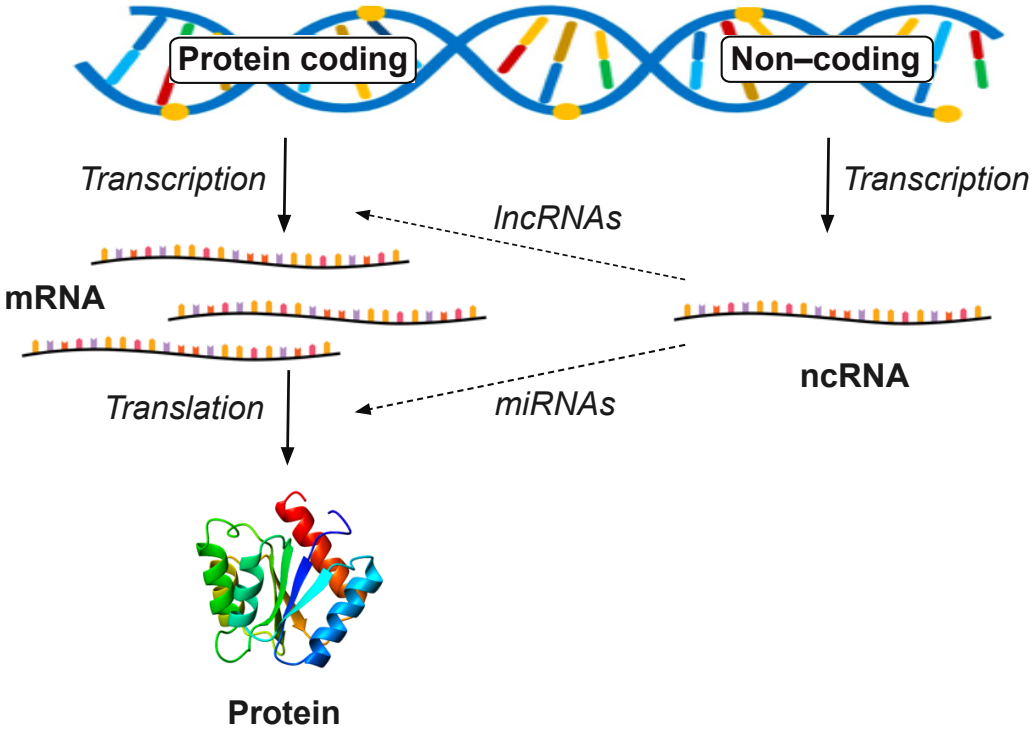

Figure 6. LncRNAs are novel modulators of transcription. LncRNA transcripts are thought to interfere with the expression of protein coding genes at the transcriptional level, whereas miRNAs are thought to silence the expression of genes at the translational level.

1.3.1. Long non-coding RNAs

LncRNAs are defined as endogenous cellular RNA molecules of more than 200 nucleotides in length, that lack an open reading frame (ORF) of significant length (less than 100 amino acids) and contain 2 or more exons (Mendell et al., 2004; Gutschner and Diederichs, 2012). They were originally discovered in mice via large-scale sequencing of full-length cDNA libraries during the FANTOM project (Okazaki et al., 2002), and since then it has become clear that mammalian genomes encode numerous lncRNAs.

Currently there are 15,779 lncRNA genes and 28,468 lncRNA transcripts documented in the human genome database (version 28) of GENCODE (genome database), compared to 19,901 protein coding genes (Gencode, 2018). The total number of lncRNAs continues to increase due to the development of advanced deep sequencing technologies which contribute to the discovery of novel lncRNA genes, some of which were previously mistakenly identified as protein coding genes (Pertea and Salzberg, 2010). Interestingly, a study by Djebali *et al.* identified protein-coding transcripts to accumulate primarily in the cytosol, whereas lncRNAs are more enriched in the nucleus (Djebali et al., 2012).

As with protein-coding genes, the majority of lncRNAs appear to be transcribed by RNA polymerase II (RNAPII) (Guttman et al., 2009). There are a few exceptions that are transcribed by RNA polymerase III (RNAPIII) including the short interspersed element (SINE) B2 RNA which was found to repress RNAPII transcription (Allen et al., 2004). As with mRNAs, lncRNAs have also been found to be subjected to transcriptional editing such as splicing, polyadenylation and 5' capping (Guttman et al., 2009). Subsequently, each lncRNA develops a final stable structure which shapes its unique cellular function and enables it to interact with other molecules (Blythe et al., 2016).

Despite the rapid increase in data relating to lncRNAs, little is known regarding their exact functions, mechanism of action or even how many different types of lncRNAs exist. Even though these transcripts are generally poorly evolutionary conserved (Necsulea et al., 2014), it is now evident that they play an important role in multiple biological pathways including the modulation of

developmental processes and pathophysiological states (Esteller et al., 2011; Chen et al., 2017b).

1.3.2. LncRNA classification

A convenient way to classify lncRNAs is based upon their position relative to well-established markers such as protein-coding genes **(Figure 7).** However, several lncRNAs do not fit into any of these categories as they present a combination of these qualities or they cover long genomic distances (Kung et al., 2013). The most significant lncRNA classes will be discussed below.

1.3.2.1. Antisense lncRNAs

Antisense lncRNAs, also known as natural antisense transcripts or NATs, are transcribed across the exons of protein-coding genes from the opposite strand, with varying degrees of overlap from partial to complete. Gene regulation by antisense transcripts occurs mainly in *cis* (Magistri et al., 2012), where the antisense lncRNA interacts with its associated or neighbouring genes. GENCODE currently lists 5,501 antisense lncRNA genes and 11,161 transcripts (Gencode, 2018). Interestingly, it is suggested that as much as 70% of protein coding genes have antisense counterparts (Faghihi and Wahlestedt, 2009; Villegas and Zaphiropoulos, 2015).

1.3.2.2. Long intergenic non-coding RNAs

Long intergenic non-coding RNAs (lincRNAs) are considered the largest and most significant group of lncRNAs, constituting approximately half the overall number of lncRNAs. GENCODE currently lists 7,490 lincRNA genes giving rise to 13,598 lincRNA transcripts (Gencode, 2018). They are stand-alone transcripts that are located between protein coding genes and can regulate gene expression by acting either in *cis* or in *trans*. Prior to the advent of sequencing, lincRNAs were originally identified using two markers of active transcription: trimethylation of lysine 4 of histone H3 (H3K4me3) and trimethylation of lysine 36 of histone H3 (H3K36me3), present at their promoter during RNAPII transcription (Guttman et al., 2009). LincRNAs appear to have undergone rapid evolution and show variable conservation

across species (Ransohoff et al., 2018). In a study by Ulitsky *et al*. mammalian lincRNA orthologues were found for just 5.1% of zebrafish lincRNA genes, demonstrating poor overall conservation when compared to protein coding genes (Ulitsky et al., 2011). Cabili *et al*. characterised the expression of human lincRNAs across 24 cell types and tissues using RNA-seq. LincRNAs were found to have lower expression levels, fewer exons and to be expressed in a cell-specific manner compared to mRNAs. LincRNA loci were typically found on average within 40kb of protein coding genes (Cabili et al., 2011). Interestingly, some of the best characterised and well-studied lincRNAs such as XIST (X inactive specific transcript) and HOTAIR (HOX transcript antisense RNA) have been shown to be important epigenetic regulators. These chromatin-associated lincRNAs were shown to mediate protein coding gene expression by recruiting and directing chromatin-modifying complexes to specific DNA regions leading to epigenetic modifications (Ulitsky and Bartel, 2013).

1.3.2.3. Enhancer RNAs

Enhancer RNA (eRNA) transcripts are found in both polyadenylated or non-polyadenylated forms and are reported to be bi-directionally expressed at active enhancer regions of the genome (Natoli and Andrau, 2012). Enhancers are DNA areas located near protein coding genes which contribute to the initiation of transcription by promoting the binding of transcription factors and other co-factors. Notably, a study by Kim *et al*. (Kim et al., 2010) revealed RNAPII-mediated transcription of eRNAs from enhancer regions in the presence of histone H3 monomethylated at lysine 4 (H3K4me1) which correlated with the activity of mRNA synthesis. As such, eRNAs are mainly thought to be *cis*-acting lncRNAs which control promoter and enhancer interactions as well as chromatin structures; resulting in the regulation of gene expression by promoting transcription of neighbouring genes (Chen et al., 2017a; Liu, 2017). Hence, eRNAs synthesis and enhancer activity are thought to be strongly correlated in regulating the transcriptional activity of neighbouring genes.

1.3.2.4. Intronic RNAs

Intronic lncRNAs are entirely transcribed from the introns of annotated protein coding genes in either a sense or antisense direction. These lncRNAs have been associated with the nesting of small ncRNAs such as miRNAs and snoRNAs as well as circular non-coding RNAs (circRNAs) (Zhang et al., 2013). In a study by Ayupe *et al*. intronic RNAs showed evidence of RNAP-II-mediated transcription and 5'-cap modifications (Ayupe et al., 2015). The functions of intronic lncRNAs remain largely unclear as they are a relatively unexplored class of lncRNAs and further investigation into their mechanisms of regulation is necessary. However, it has been suggested that intronic lncRNAs are often co-transcribed with their host protein coding gene, thus possibly sharing strong regulatory features and relationships with their host gene (Boivin et al., 2018).

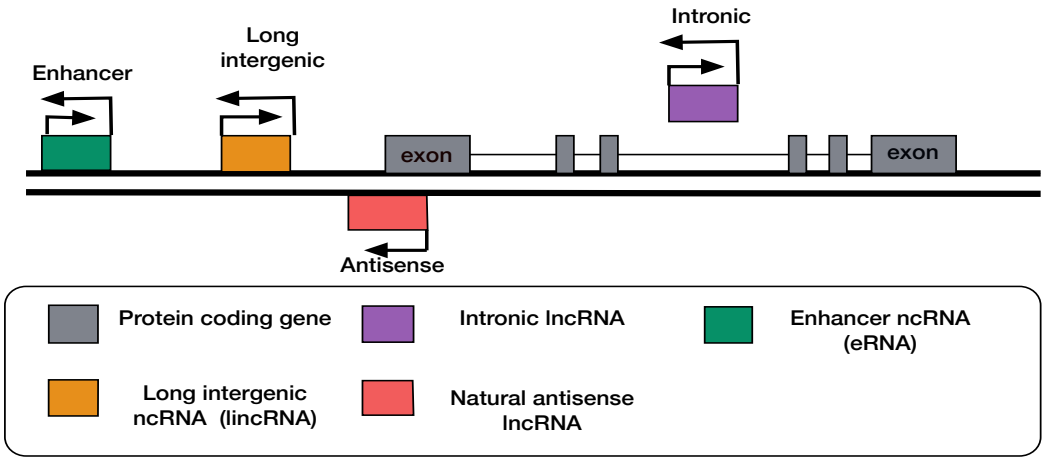

Figure 7. Classification of the most widely found lncRNAs according to their genomic location. Attempts to resolve the transcriptomic complexity of lncRNAs have led to their classification based on their genomic proximity to protein coding genes. LincRNAs and eRNAs are stand-alone transcription units situated near protein coding genes. Intronic lncRNAs are found within the introns of protein coding genes, while antisense lncRNAs are transcribed in the opposite sense of the exonic regions of protein coding genes.

1.3.2.5. circRNAs

circRNAs are a class of recently discovered regulatory RNAs which were found to interact and regulate the activity of miRNAs, hence usually referred to as 'miRNA sponges' (Ebert et al., 2007; Hansen et al., 2011). A study by Memczak *et al.* identified approximately 2,000 human, 1,900 mouse and 700 nematode circRNAs that may be expressed from both coding and non-coding genomic loci (Memczak et al., 2013). circRNAs have been found to localise primarily in the cytoplasm and inhibit miRNAs by acting as miRNA-competing transcripts (Jeck et al., 2013; Hansen et al., 2013). Interestingly, another type of circRNAs, known as circular intronic RNAs (ciRNAs) were shown to be located in the nucleus acting as *cis*-regulators of RNAPII-mediated transcription and expression of parent genes (Zhang et al., 2013).

1.3.2.6. Pseudogenes

The non-coding genome also gives rise to pseudogenes which are derived from protein coding genes that lose their coding potential through evolution (Balakirev and Ayala, 2003). There are currently 14,723 pseudogenes annotated in GENCODE (version28). However, it appears that a number of pseudogenes may potentially regulate the expression of protein coding genes by processing into short interfering RNAs or acting as miRNA decoys (sponges) to regulate oncogenes during cancer progression (Pink et al., 2011). Pseudogenes were also reported to be co-transcribed with their parent gene, function as antisense transcripts or even produce short peptides (Li et al., 2013).

1.3.3. Characteristics of lncRNAs

LncRNAs present several distinct characteristics when compared to mRNAs regarding their size, specificity, organisation and subcellular localisation. However, despite the differences, they also possess a lot of similarities to mRNAs regarding their biogenesis and form.

1.3.3.1. LncRNAs and mRNAs share similar biogenesis pathways

LncRNAs are predominantly transcribed by RNAPII and most are spliced, polyadenylated at the 3'-end, 5'-end capped with 7-methylguanosine and are associated with similar histone markers to those of mRNAs (Guttman et al., 2009; Hon et al., 2017; Derrien et al., 2012). Non-polyadenylated lncRNAs, including those associated with enhancer regions (Natoli and Andrau, 2012), are thought to be stabilised through other mechanisms. These include ribonuclease P (RNase P) cleavage to generate mature 3'-ends, the formation of circular molecular structures such as circRNAs or capping by small nucleolar ribonucleoproteins (snoRNP) complexes (Memczak et al., 2013; Zhang et al., 2014b). Unlike mRNAs which are known for their protein coding functions and translational potential, lncRNAs lack an ORF of significant length and quality (Derrien et al., 2012) and are therefore deemed to have no translational capacity. However, interestingly a study by Ruiz-Orera *et al.* suggested that lncRNAs may give rise to small novel peptides (Ruiz-Orera et al., 2014).

1.3.3.2. LncRNAs are expressed at lower levels compared to mRNAs

LncRNAs are generally shorter in length, have fewer but longer exons and are expressed at lower levels compared to mRNAs (Cabili et al., 2011; Derrien et al., 2012; Hezroni et al., 2015). Indeed, lncRNAs demonstrated lower expression in all tissues compared to mRNAs except in the testes where they showed tissue-specific elevated expression levels (Cabili et al., 2011; Melé et al., 2017; Necsulea et al., 2014; Ransohoff et al., 2018). These lower expression levels were initially advanced as evidence that lncRNAs were simply the result of transcriptional 'noise'. This has since been disproved following evidence showing biological functionality (see Section 1.3.4).

1.3.3.3. LncRNAs expression is cell and tissue specific

Transcriptome-wide studies demonstrated that expression of lncRNAs is specific for the cell, tissue, developmental or disease state, as well as highly

dependent on context and time (Derrien et al., 2012; Ulitsky and Bartel, 2013; Melé et al., 2017; Cabili et al., 2011). For example, Cabili *et al*. found that 78% of lincRNAs were tissue-specific across 24 tissue and cell types compared to approximately 19% of protein coding genes (Cabili et al., 2011). This specificity of lncRNA expression may explain their low levels of expression compared to mRNAs and indicates that they possess cell/tissue selective functions (Gloss and Dinger, 2016).

1.3.3.4. LncRNAs show poor evolutionary conservation

Unlike protein coding genes, lncRNAs generally show poor evolutionary conservation (Derrien et al., 2012; Cabili et al., 2011; Necsulea et al., 2014) and this lack of sequence conservation has made it difficult to identify functional domains and to compare their biological significance across species (Rands et al., 2014). In general, the exon regions of lincRNAs were shown to demonstrate higher conservation than random un-transcribed intragenic regions although still considerably less than that observed in exon regions of protein coding genes. Interestingly, the conservation across the promoter regions of lncRNAs is comparable to that of protein coding genes (Guttman et al., 2009; 2010). This lack of conservation appears to be related to the rapid evolution of lincRNAs, most lincRNAs have no conserved orthologues (Hezroni et al., 2015). In contrast to sequence conservation, thousands of lncRNAs were found to demonstrate highly conserved genomic positions (synteny) (Ulitsky et al., 2011). Despite the general observation of poor conservation, a small number of lncRNAs demonstrate high conservation at both sequence and structure level and include well-characterised lincRNAs such as MALAT1 (metastasis associated lung adenocarcinoma transcript 1) and NEAT1 (nuclear enriched abundant transcript 1) (Johnsson et al., 2014).

1.3.3.5. Subcellular localisation of lncRNAs

After transcription in the nucleus, mRNA transcripts tend to be transported to the cytoplasm where they undergo translation. In contrast, lncRNAs are found both in the nucleus and the cytoplasm although current evidence suggests that are predominantly enriched in the former (Derrien et al., 2012; Djebali et

al., 2012). Nuclear lncRNAs include some of the best studied lncRNAs such as NEAT1 (Clemson et al., 2009), MALAT1 (Tripathi et al., 2010) and XIST (Brown et al., 1992) where they are thought to regulate epigenetic modifications and mRNA processing. However, even though lncRNAs as a group are more enriched in the nucleus compared to mRNAs, cytoplasmic lncRNAs are reported to be expressed in higher numbers (Ulitsky and Bartel, 2013; Rashid et al., 2016). Interestingly, a report by van Heesch *et al.* (van Heesch et al., 2014) showed a 30% enrichment of lncRNAs in the cytoplasm and 38% in ribosomal fractions compared to just 17% in the nucleus. Additionally, ribosome-profiling experiments have found abundant numbers of lncRNAs associated with ribosomes, suggesting they may actually be translated (Ingolia et al., 2011; Ruiz-Orera et al., 2014). However, further studies failed to detect protein products from the supposed translation of lncRNA ORFs, suggesting that ribosomes can distinguish between coding and non-coding transcripts and concluding that lncRNAs are unlikely to encode peptides/proteins (Quinn and Chang, 2016).

1.3.4. Biological significance of lncRNAs

LncRNAs are a relatively novel class of RNA molecules, hence their functions remain largely unexplored. They were originally considered transcriptional 'noise' and although the majority are still likely to be biologically inactive there is emerging evidence that a substantial number have functional activity. In particular, a number of highly expressed lncRNAs such as HOTAIR (Gupta et al., 2010), XIST (Brown et al., 1992; Cerase et al., 2015), MALAT1 (Tripathi et al., 2010), and H19 (Raveh et al., 2015) have been studied in more depth and are relatively well-characterised. Nuclear located lncRNAs are generally associated with chromatin modifications, transcriptional regulation and RNA processing, whereas cytoplasmic lncRNAs have been linked with mRNA stability/translation and as direct agonists/antagonists of protein expression **(Figure 8)**. As such, lncRNA transcripts have been associated with the regulation of all aspects of mRNA processing and protein activity (Zhang et al., 2014a).

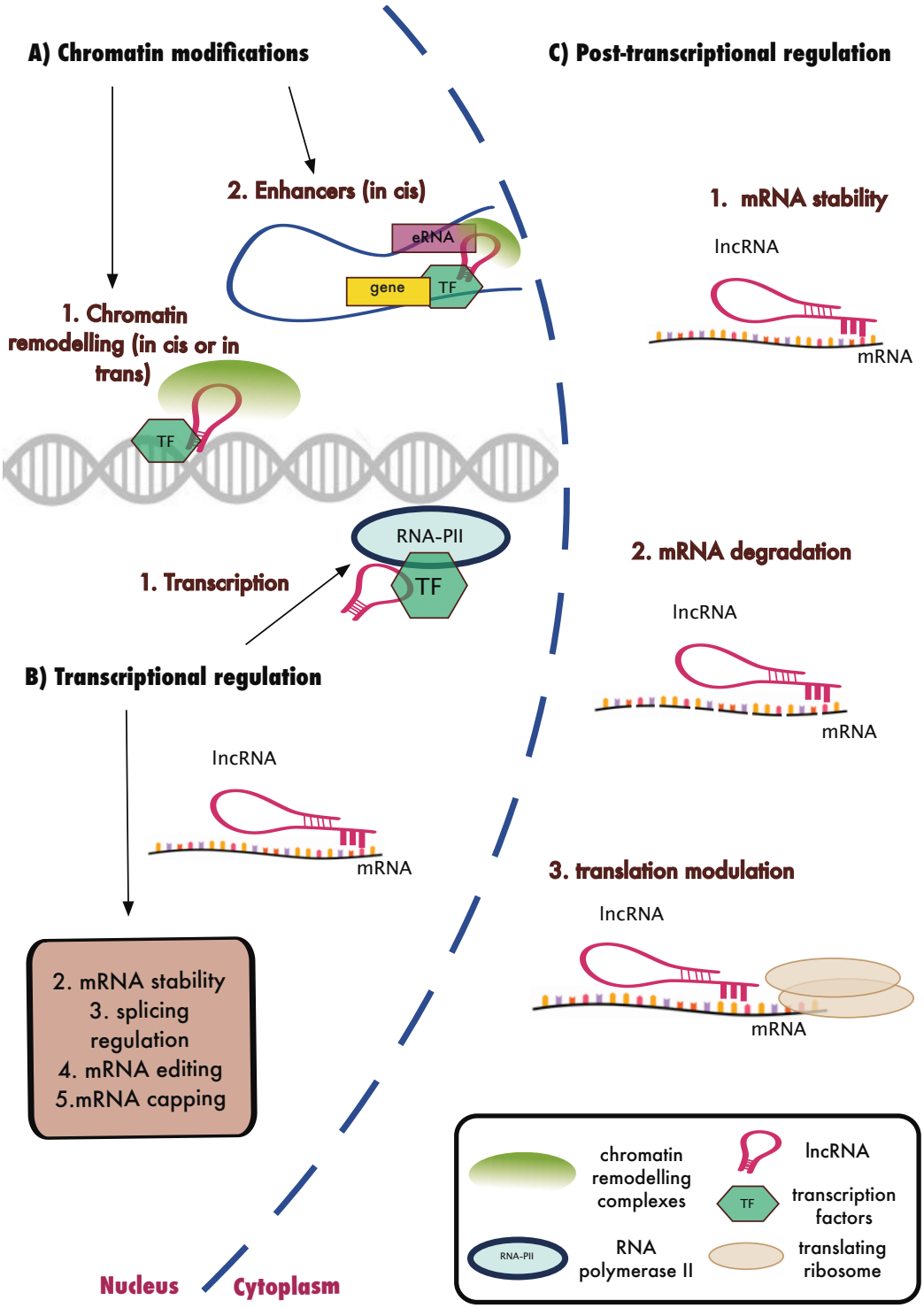

Figure 8. Biological functions of lncRNAs. LncRNAs were shown to interact with protein coding genes and their transcripts to regulate gene expression. (A) Nuclear lncRNAs interact with chromatin remodelling factors and transcription factors to regulate the expression of neighbouring or distal genes. (B) Nuclear lncRNAs also regulate transcription and several other transcriptional events of RNA processing. (C) Cytoplasmic lncRNAs were shown to interfere with post-transcriptional regulation such as mRNA stability and degradation as well as translational regulation of mRNAs.

1.3.4.1. Chromatin modifications

LncRNAs have been implicated in the regulation of epigenetic changes through recruiting and guiding chromatin remodelling complexes to specific genomic loci both in *cis* and in *trans* to regulate transcription **(Figure 9**). The chromatin remodelling complexes may exert their effects by repressing or activating the expression of protein coding genes via the recruitment of chromatin-modifying factors such as the Polycomb repressive complexes (PRC1 and PRC2) and histone methyltransferases (Saxena and Carninci, 2011; Han and Chang, 2015).

For example, one of the first lncRNAs to be characterised was the mammalian *cis*-acting XIST which mediates the silencing of one X chromosome in females during development. XIST induces the formation of repressive chromatin and the recruitment of PRC proteins (PRC2) to completely inactivate one of the two X chromosomes by dosage compensation during the early embryonic development of females. Interestingly, other lncRNAs have been shown to interact with and regulate the expression of XIST, with the most prominent being its own natural antisense lncRNA (TSIX) (Pontier and Gribnau, 2011; Froberg et al., 2013; Lee and Bartolomei, 2013). Another well-studied lncRNA is HOTTIP (HOXA Distal Transcript Antisense RNA) which was found to promote the expression of the HOXA gene. HOTTIP directly interacts with adaptor protein WDR5 and the mixed-lineage leukaemia protein 1 (MLL1) histone lysine methyltransferase complex (also known as Histone-lysine N-methyltransferase 2A) to recruit them to the HOXA locus through chromatin looping, causing H3K4 trimethylation and inducing the transcription of HOXA (Wang et al., 2011).

LncRNAs can also migrate from their site of transcription and regulate the expression of genes in *trans* either distally located on the same chromosomes or on different chromosomes. Such a lncRNA is the well-studied antisense intergenic lncRNA HOTAIR which is transcribed from the HOXC locus (Gupta et al., 2010). HOTAIR is thought to silence the transcription of the distant HOXD gene by acting as a scaffold for the recruitment of the repressive

chromatin PRC2 complex leading to H3K27 trimethylation and H3K4 demethylation (Kugel and Goodrich, 2012; Hajjari and Salavaty, 2015).

LncRNAs have also been associated with the regulation of the monoallelic expression (only one of the two copies of a gene is expressed) of genes according to their parents of origin, also known as genomic imprinting. Imprinted control regions may be associated with the expression of lncRNAs, such as the paternally expressed Airn lncRNA, also known as IGF2R-AS1, which was shown to silence the maternally expressed genes Igf2r/Slc22a2/Slc22a3 (Sleutels et al., 2002). The imprinted lncRNA clusters are thought to silence the expression of neighbouring genes in *cis* by recruiting chromatin-modifying complexes and maintaining repressive DNA methylation at adjacent loci (Kanduri, 2016).

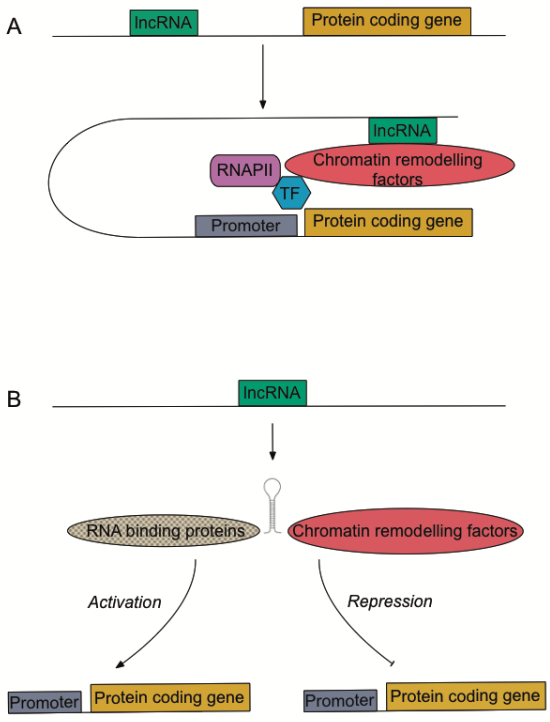

Figure 9. LncRNAs function in cis and in trans to regulate gene expression. LncRNAs interact with chromatin remodelling factors and binding proteins in order to regulate the expression of (A) neighbouring genes, in *cis* or (B) in *trans*, distally located genes that could be located on the same or a different chromosome.

1.3.4.2. Transcriptional regulation

The process of transcription and the associated RNA processing and organisation of the nuclear architecture, may also be regulated by lncRNA transcripts. LncRNAs have been shown to regulate transcription factors and the RNAP-II transcription machinery, leading to an increase or suppression of transcription, as well as mRNA processing mechanisms including splicing, capping and editing (Geisler and Coller, 2013). For example, the lncRNA MALAT1 is found in nuclear speckles and is thought to regulate alternative splicing of mRNAs (Tripathi et al., 2010). MALAT1 may act as a scaffold by guiding serine/arginine (SR) splicing factors to sites of transcription where splicing takes place (Kopp and Mendell, 2018). Another example is the natural antisense transcript ZEB2 (NAT) which was shown to regulate the expression of the Zeb2 gene via splicing of an internal ribosome entry site (IRES) located within an intron in the 5-untranslated region (UTR) of the Zeb2 gene (Beltran et al., 2008).

1.3.4.3. Post-transcriptional regulation

Several lncRNAs are transported from the nucleus to the cytoplasm where they are thought to be involved in regulating mRNA stability and translation. As an example, the ubiquitin carboxy-terminal hydrolase L1 antisense RNA 1 (Uchl1-as1) exhibits positive regulation of translation of the Uchl1 protein through the embedded inverted SINEB2 element, although its exact mechanism of action is unclear (Carrieri et al., 2012; Podbevšek et al., 2018). In contrast, the lincRNA-p21 (also known as tumour protein p53 pathway core-pressor 1) was shown to inhibit the translation of target mRNAs by negatively regulating the translation of CTNNB1 (β-catenin) and JUNB (transcription factor jun-b) (Yoon et al., 2012). LncRNAs such as 1/2-sbsRNAs and gadd7 have also been shown to regulate mRNA stability through interactions with various proteins including Staufen1 (STAU1) and cyclin-dependent kinase 6 (Cdk6), respectively (Gong and Maquat, 2011). Additionally, lncRNAs such as the natural antisense BACE1-AS and TINCR were shown to enhance mRNA stability *in vitro* (Zhang et al., 2014a; Rashid et al., 2016). Interestingly, lncRNAs may also regulate mRNA expression by binding to specific miRNAs

where they function as competing endogenous RNAs (ceRNAs). ceRNAs were shown to protect target mRNAs from repression by acting as 'miRNA sponges', presenting another post-transcriptional regulatory role of lncRNAs (Cesana et al., 2011; Salmena et al., 2011; Zhang et al., 2014a).

1.3.5. LncRNAs and the regulation of lung fibrosis

Our knowledge of the role of lncRNAs in IPF is currently very limited. A microarray study by Cao *et al*. using the bleomycin-induced lung fibrosis rat model identified differential expression levels of multiple lncRNAs and mRNAs, most significantly lncRNAs AJ005396 and S69206 (Cao et al., 2013). A subsequent study using the bleomycin model focused on two differentially expressed lncRNAs, MRAK088388 and MRAK081523 and reported that these could regulate expression of protein coding genes by acting as ceRNAs (sponges) for miR-29b-3p and let-7i-5p (Song et al., 2014). In a similar vein, Huang *et al*. identified 34 lncRNAs with potential miRNA binding sites suggesting possible lncRNA-miRNA interactions. Real-time polymerase chain reaction (RT-PCR) confirmed the expression of these lncRNAs in human IPF lung tissue and 9 of them were dysregulated in IPF. Knockdown of lncRNAs CD99P1 and n341773 indicated that these might regulate lung fibroblast differentiation and proliferation (Huang et al., 2015). More recently, sequencing and microarray analysis of mouse fibrotic lungs identified 513 up-regulated and 204 down-regulated lncRNAs, of which uc.77 and 2700086A05Rik were found to mediate changes in EMT when overexpressed (Sun et al., 2016). Wu *et al.* has demonstrated that silica-induced pulmonary fibrosis may be inhibited by miR-489 in mice. miR-489 was also shown to suppress fibroblast differentiation and inflammation by targeting Smad3 and MyD88, respectively. Interestingly, the upregulation of the lncRNA CHRF (cardiac hypertrophy-related factor) was found to reverse the inhibitory effects of miR-489 in mice, as well as in macrophage and fibroblast cell lines. This suggests that CHRF may play a role in the regulation of miR-489 and the activation of the inflammation and fibrotic signalling pathways (Wu et al., 2016). The expression of lncRNA H19 has also been implicated in the development of pulmonary fibrosis in a study by Tang *et al*. (Tang et al., 2016).

LncRNA H19 was found to interact with miR-29b and to exert pro-fibrotic effects by regulating the expression of collagen and α-smooth muscle actin in the bleomycin model of fibrosis. However, another study demonstrated upregulated expression of lncRNA H19 in the bleomycin mouse model as well as in fibroblast cell lines following TGF-β1 exposure (Lu et al., 2018). LncRNA H19 was found to act as a ceRNA for miR-196a to regulate collagen expression. Moreover, the elevated expression of lncRNA AP003419.16 in the peripheral blood of IPF patients has been linked with the increased risk of developing age-associated IPF (Hao et al., 2017). Interestingly, the expression of lncRNA NONMMUT065582, also known as pulmonary fibrosis-associated RNA (PFAR), was found to be elevated in the fibrotic lungs of mice as well as mouse fibroblasts (Zhao et al., 2018). LncRNA PFAR was shown to promote the development of fibrosis by acting as ceRNA for miR-138 and regulating the expression of yes-associated protein 1 (YAP1).

Table 1. LncRNAs associated with pulmonary fibrosis

LncRNA	**Effect on fibrosis**	**Research model**	**Reference**
AJ005396, S69206	Pro-fibrotic	Bleomycin-induced fibrosis rat model	(Cao et al., 2013)
MRAK088388, MRAK081523	Pro-fibrotic	Bleomycin-induced fibrosis rat model	(Song et al., 2014)
CD99P1, n341773	Pro-fibrotic	Human lung tissue	(Huang et al., 2015)
uc.77, 2700086A05Rik	Pro-fibrotic	Paraquat-induced fibrosis mouse model	(Sun et al., 2016)
lncRNA CHRF	Pro-fibrotic	Silica-induced fibrosis mouse model	(Wu et al., 2016)
LncRNA H19	Pro-fibrotic	Bleomycin-induced fibrosis mouse model	(Tang et al., 2016)
LncRNA H19	Pro-fibrotic	Bleomycin-induced fibrosis mouse model, human fibroblast cell line MRC-5	(Lu et al., 2018)
AP003419.16	Pro-fibrotic	Human blood samples	(Hao et al., 2017)
PFAR	Pro-fibrotic	Bleomycin-induced fibrosis mouse model, primary mouse fibroblasts	(Zhao et al., 2018)

1.3.6. LncRNAs in other types of fibrosis

As with IPF, the role of lncRNAs in the development of other types of fibrosis including that observed in the heart, liver and kidney is currently under investigation.

In cardiac fibrosis, the cardio-protective lncRNA, myosin heavy chain-associated RNA transcript (Mhrt) was found to bind to the helicase domain of the chromatin-remodelling factor BRM/SWI2-related gene 1 (Brg1) and thereby prevent DNA binding. Through inhibiting the action of Brg1, this lncRNA was shown to protect the heart from fibrosis and remodelling (Han et al., 2014). As observed in the lung, CHRF has also been associated with the regulation of cardiac hypertrophy by targeting miR-489 and regulating MyD88 expression (Wang et al., 2014). In addition, the circulating lncRNA LIPCAR (long intergenic non-coding RNA predicting cardiac remodelling) has been proposed as a novel biomarker of cardiac remodelling (Kumarswamy et al., 2014). Expression of lncRNA-H19 was found to be up-regulated in cardiac fibroblasts and fibrotic tissues and to play a role in proliferation and fibrosis, potentially through the regulation of DUSP5 (Dual specificity protein phosphatase 5) (Tao et al., 2016). More recently, knockdown of a lncRNA named myocardial infarction associated transcript (MIAT) was shown to attenuate fibrosis by decreasing collagen production and proliferation in cardiac fibroblasts. Mechanistic studies showed that MIAT acted as a ceRNA (sponge) for the miR-24 (Qu et al., 2017).

In the case of hepatic fibrosis, the maternally expressed gene 3 (MEG3) may possess an important role in fibrosis as it was found to be downregulated in the CCL_4–induced liver fibrosis model and human fibrotic liver tissues (He et al., 2014). Although its role in hepatic fibrosis is still unclear, exposure of human hepatic stellate cell lines (HSC) LX-2 cells to TGF-β1 resulted in decreased expression of MEG3 and an increase in α-SMA expression. More importantly, methylation of the MEG3 promoter and subsequent expression of MEG3 were found to be regulated by DNA methyltransferase 1 (DNMT1),

while the depletion of DNMT1 was shown to affect α-SMA and COL1A1 mRNA and protein expression in TGF-β1-treated hepatic stellate LX-2 cells (He et al., 2014). LincRNA-21 demonstrated reduced expression in mice hepatic fibrosis models and was shown to regulate primary HSC activation by promoting p21 (cyclin-dependent kinase inhibitor 1) expression, leading to inhibition of the cell cycle and proliferation *in vitro* (Zheng et al., 2015). Another study by Yu *et al.* demonstrated elevated expression of lncRNA APTR (Alu-mediated p21 transcriptional regulator) in hepatic fibrotic tissues and TGF-β1 activated HSC. Knockdown of APTR was shown to inhibit fibrosis *in vivo* and activation of HSC, as well as increase the expression of p21 and inhibit cell cycle progression and proliferation (Yu et al., 2015a). LncRNA growth arrest-specific transcript 5 (GAS5) was also found to play a role in hepatic fibrogenesis with reduced expression in mouse, rat, human fibrotic liver tissue and in activated HSCs. GAS5 was shown to be primarily located in the cytoplasm, acting as a ceRNA for miR-222 and reducing the activation and proliferation of HSCs (Yu et al., 2015b).

The role of lncRNAs in renal fibrosis also remains largely unexplored. A recent study by Xie *et al.* showed that lncRNA-H19 expression is upregulated in renal fibrosis in both TGF-β2-induced HK-2 cell fibrosis *in vitro* and during unilateral ureteral obstruction (UUO)-induced renal fibrosis *in vivo*. LncRNA-H19 knockdown was shown to attenuate fibrosis via interactions with miR-17 and fibronectin, suggesting that inhibition of LncRNA-H19 may potentially present a novel anti-fibrotic treatment (Xie et al., 2016). In a different study, RNA sequencing identified 151 lncRNAs associated with the TGF/Smad3 signalling pathway using the UUO-induced mouse model of renal fibrosis (Zhou et al., 2014). Arvaniti *et al.* identified numerous genes that were differentially expressed in the UUO renal fibrosis mouse model including several lncRNAs; the functional roles of RP23-45G16.5, 3110045C21Rik and AI662270 lncRNAs were also investigated. Overexpression of RP23-45G16.5 in mouse kidney epithelial cells (M-1 cell line) was shown to upregulate CCL2 mRNA levels while 3110045C21Rik overexpression was found to elevate CDH1 (e-cadherin) and inhibit α-SMA and TGFB1 mRNA expression. AI662270

lncRNA failed to demonstrate any significant effect on expression of these genes (Arvaniti et al., 2016).

1.3.7. miRNAs

LncRNAs may also give rise to miRNAs which can either be embedded within their exons or intron regions; thus many are named after the lncRNA host gene from which they are encoded (Dykes and Emanueli, 2017).

miRNAs are commonly evolutionarily conserved and are the most well studied class of ncRNAs. They are 20-25 nucleotide long single–stranded molecules that are thought to modulate gene expression at the post-transcriptional level via the RNA interference pathway (O'Reilly, 2016). Primary miRNAs are transcribed in the nucleus by RNAP-II and these capped and polyadenylated transcripts are then processed by Drosha, a RNAse III ribonuclease, into precursor miRNAs. They are then transported from the nucleus into the cytoplasm by exportin 5 through the nuclear pore complexes for further processing by DICER, a second RNAse III endonuclase, to give rise to mature double stranded miRNAs. The mature miRNA is incorporated into the RNA–induced silencing complex (RISC) where the mature miRNA strand (guide strand) binds to a member of the Argonaute (ago) protein family complex that mediates gene silencing by either repression of mRNA translation or reduction in mRNA stability, following binding within the 3' untranslated region (3'UTR) of target mRNAs. The other strand (passenger stand) is usually degraded, however in rare cases it remains functional (Jiang et al., 2010; Vettori et al., 2012). Since their initial discovery in *Caenorhabditis elegans* in 1993 (Lee et al., 1993), miRNAs have received remarkable research interest and it is now evident that they play a crucial role in the regulation of multiple cellular processes and their aberrant expression is implicated in various human diseases (Li and Kowdley, 2012). miRNAs have been associated and shown to interfere with several crucial cellular processes such as proliferation, differentiation and apoptosis (Carleton et al., 2007).

Many miRNAs are now associated with the development and progression of

fibrosis with evidence suggesting that they directly regulate multiple molecules within the pro-fibrotic pathways including TGF signalling, collagen deposition or ECM synthesis. As such, miRNAs are considered an attractive therapeutic target using locked nucleic acid (LNA) antisense technology to antagonise their function (Jiang et al., 2010; O'Reilly, 2016). Several antagomirs such as anti-miR-26a (Liang et al., 2014a) and let-7d (Pandit et al., 2010) have been used to block the functions of various miRNAs associated with fibrosis both *in vivo* and *in vitro*. However, only the anti- miRNA-122 oligonucleotide is currently in clinical use for the treatment of hepatitis C infection, marketed as Miravirsen (Janssen et al., 2013).

1.3.7.1. miRNAs and their role in IPF

IPF may also be linked to changes in the expression of miRNAs, with a report showing that approximately 10% of miRNAs are differentially expressed in IPF patients (Pandit et al., 2011). miRNAs have been proposed to mediate pulmonary fibrotic processes by acting either as positive or negative entities exerting pro-fibrotic or anti-fibrotic effects respectively.

There are numerous miRNAs associated with the development of IPF, however miRNAs such as miR-29, miR-26α, miR-21 and Let-7d have attracted significantly more interest and their mechanism of action is better understood. Overexpression of miRNAs such as miR-199a-5p and miR-21 were shown to promote myofibroblast differentiation induced by TGF-β1 in lung fibroblasts (Lino Cardenas et al., 2013; Liu et al., 2010). Upregulation of miR-145 is also seen in IPF lungs and following TGF-β1 activation in human lung fibroblasts (Yang et al., 2013). Several other miRNAs were also shown to promote fibrosis such miR-424, miR-210, miR-96 and miR-154; leading to TGF-β1-induced EMT in lung epithelial cells (miR-424), increasing hypoxia-induced lung fibroblast proliferation (miR-210), enhancing the proliferative and anti-apoptotic phenotype in IPF fibroblasts (miR-96) and stimulating the proliferation and migration of lung fibroblasts (miR-154) (Xiao et al., 2015; Bodempudi et al., 2014; Nho et al., 2014; Milosevic et al., 2012).

Interestingly, anti-fibrotic miRNAs appear down-regulated in IPF lungs including the miR-29 family, which was shown to be further suppressed upon

TGF-β1 exposure in human fibroblasts (Cushing et al., 2011). A study by Xiao *et al*. suggests that miR-29 is a downstream target gene of Smad3 and negatively regulated by TGF-β/Smad signalling in pulmonary fibrosis (Xiao et al., 2012). miR-29 has also been shown to inhibit the expression of ECM components in fibroblasts grown on IPF-derived ECM (Parker et al., 2014). miR-26α and let-7d are also down-regulated in pulmonary fibrosis and are further suppressed by TGF-β1 in a Smad3-dependent manner (Pandit et al., 2010; Liang et al., 2014a). miR-26α and let-7d may mediate EMT in IPF via the transcriptional factor HMGA2 (Pandit et al., 2010; Liang et al., 2014b). miR-326 is another miRNA thought to impact upon the lung fibrotic response via regulation of the TGF-β1 signalling pathway and several other pro-fibrotic genes (Das et al., 2014).

1.4. Conclusion

It has become evident that our understanding of the complex mechanisms that regulate gene expression are still at an early stage. In particular, the advent of high-throughput sequencing technologies has revolutionised our ability to examine the genome and transcriptome under physiological and pathological conditions. One of the newest families of regulatory elements to be identified are the long non-coding RNAs, with emerging evidence that these regulate multiple biological responses and that changes in their expression may be related to the development of disease. Indeed, there is now evidence that lncRNAs are dysregulated in several types of cancer, in the immune response as well as neurological disorders (Schmitt and Chang, 2016; Clark and Blackshaw, 2014; Chen et al., 2017c).

IPF is a fatal progressive chronic disease characterised by scar tissue accumulation in the lungs leading to impaired gas exchange and restricted ventilation. Previous studies have identified several miRNAs that may play an important role in the development of fibrosis. In contrast, little is known about the role of lncRNAs in the pathogenesis of IPF and it is this question that was addressed as part of this thesis.

1.5. Hypothesis

Although IPF is a complex disease with an unknown aetiology, several key cells including lung epithelial cells and fibroblasts have been implicated in the initiation and progression of the disease. We hypothesise that “lncRNAs regulate the function of human fibroblasts in the normal and IPF lungs”.

To examine this hypothesis, we addressed the following aims and compiled the resulting data into three publications:

1. Identified lncRNAs that were differentially expressed between normal and IPF lung biopsies (Chapter 2)
2. Identified lncRNAs that were differentially expressed between normal and IPF lung fibroblasts (LINC00960 and LINC01140) (Chapter 2)
3. Identified lncRNAs that were differentially expressed following TGF-β1-induced activation of control and IPF lung fibroblasts (Chapter 2)
4. Examined whether differences between control and IPF lung fibroblasts are reflected at the epigenetic level using a histone marker of primed promoters and enhancers, H3K4me1 (Chapter 2)
5. Compared the TGF-β1-induced fibrotic response, PDGF-AB-induced proliferative response and the IL-1β-induced inflammatory response between control and IPF lung fibroblasts (Chapter 2)
6. Examined the role of the differentially expressed long non-coding RNAs (LINC00960 and LINC01140) in the TGF-β1-induced fibrotic response, PDGF-AB-induced proliferative response and the IL-1β-induced inflammatory response in control and IPF lung fibroblasts (Chapter 2)
7. Identified lncRNAs that were differentially expressed following IL-1β-induced activation of control lung fibroblasts (IL7AS and MIR3142HG) (Chapter 3)

8. Examined the role of the differentially expressed lncRNAs (IL7AS and MIR3142HG) in the IL-1β-induced inflammatory response in control and IPF lung fibroblasts (Chapter 3)

9. Examined the effect of pirfenidone and nintedanib on the TGF-β1, PDGF-AB and IL-1β-induced responses of control and IPF lung fibroblasts (Chapter 4)

2. Chapter 2 – Long intergenic non-coding RNAs regulate human lung fibroblast function: Implications for idiopathic pulmonary fibrosis

Statement of Authorship Form

This declaration concerns the article entitled:

Long intergenic non-coding RNAs regulate human lung fibroblast function: Implications for idiopathic pulmonary fibrosis												
Publication status (tick one)												
draft manuscript		**Submitted**		**In review**	√	**Accepted**		**Published**				
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Candidate's contribution to the paper (detailed, and also given as a percentage).	The candidate contributed to/ considerably contributed to/predominantly executed the… Formulation of ideas (50%): Marina Hadjicharalambous and Mark A. Lindsay decided equally on direction of the project based on previous literature and research. Design of methodology (70%): Experiments were mainly designed by Marina Hadjicharalambous. Mark A. Lindsay, Benoit T. Roux, Deborah L. Clarke and Lynne Murray contributed equally (Carol A. Feghali-Bostwick provided the fibroblasts). Experimental work (100%): All experimental work was undertaken by Marina Hadjicharalambous (Eszter Csomor assisted with the array experiment). Presentation of data in journal format (90%): Marina Hadjicharalambous was the main author of the paper with corrections and suggestions offered by the co-authors.											
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.											
Signed							**Date**					

Long intergenic non-coding RNAs regulate human lung fibroblast function: Implications for idiopathic pulmonary fibrosis

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2.1. Abstract

Phenotypic changes in lung fibroblasts are believed to contribute to the development of Idiopathic Pulmonary Fibrosis (IPF), a progressive and fatal lung disease. Long intergenic non-coding RNAs (lincRNAs) have been identified as novel regulators of gene expression and protein activity. In non-stimulated cells, we observed reduced proliferation and inflammation but no difference in the fibrotic response of IPF fibroblasts. These functional changes in non-stimulated cells were associated with differential expression of H3K4me1, a histone marker of primed promoters and enhancers. Following activation with TGF-β1 and IL-1β, we demonstrated an increased fibrotic but reduced inflammatory response in IPF fibroblasts. There was no significant difference in proliferation following PDGF exposure. The lincRNAs, LINC00960 and LINC01140 were upregulated in IPF fibroblasts. Knockdown studies showed that LINC00960 and LINC01140 were positive regulators of proliferation in both control and IPF fibroblasts but had no effect upon the fibrotic response. Knockdown of LINC01140 but not LINC00960 increased the inflammatory response, which was greater in IPF compared to control fibroblasts. Overall, these studies demonstrate for the first time that lincRNAs are important regulators of proliferation and inflammation in human lung fibroblasts and that these might mediate the reduced inflammatory response observed in IPF-derived fibroblasts.

2.2. Introduction

Idiopathic pulmonary fibrosis (IPF) is a fatal progressive chronic disease characterised by scar tissue accumulation in the lungs leading to impaired gas exchange and restricted ventilation [1-3]. The etiology and pathogenesis of the disease are still unclear, although recent research has indicated that persistent epithelial injury and/or exposure to pathogens, leads to the secretion of fibrotic, proliferative and inflammatory mediators such as TGF-β1 [4], PDGF [5] and IL-1β [6] . These are then thought to act upon surrounding fibroblasts, to induce an exaggerated wound healing response that contributes towards the development and progression of IPF [1,3].

Comparison of the phenotype of lung fibroblasts derived from IPF patients with those from non-fibrotic patients has shown that these exhibit multiple differences including reduced apoptosis [7,8] and diminished capacity to synthesis cyclooxygenase 2 and prostaglandin E2 [9]. Differences have also been observed in proliferation and release of fibrotic components, although these have resulted in contradictory observations [10-13]. Attempts to understand these persistent phenotypic changes at the epigenetic level have shown differences in the pattern of DNA methylation [14,15]. However, although there are reports of histone changes associated with individual genes linked to IPF [16], there has been no attempt to determine if there are genome wide changes in the profile of histone modifications.

High-throughput sequencing indicates that much of the human genome is transcribed into non-coding RNAs (ncRNAs). The majority of ncRNAs (>90%) are involved in house-keeping activities such as translation (ribosomal RNA), splicing (short nuclear RNAs) and post-transcriptional RNA modifications (short nucleolar RNA) whilst the others are broadly classified as either short ncRNAs (<200 nt (nucleotides)) or long ncRNAs (lncRNAs) (>200 nt). The microRNA family of short ncRNAs are the best characterised and are known to induce mRNA degradation or block messenger RNA (mRNA) translation via the RNA interference pathway [17]. In contrast, little is known about lncRNAs which are commonly divided into three groups: long intergenic non-coding RNAs (lincRNAs) that are located between protein-coding genes, antisense that are transcribed across protein coding genes on the reverse strand and pseudogenes, that are non-translated versions of protein coding genes [18,19]. In most cases, it is believed that the actions of lncRNAs are mediated through domains that interact with proteins, acting as scaffolds or to modulate their activity [20,21]. At the present time, a number of miRNAs have been implicated in the regulation of fibroblast function and in the development of IPF including let-7d [22], miR-17~92 [23], miR-101 [24] and miR-155 [25]. In contrast, although there is accumulating evidence to indicate that lncRNAs are important regulators of biological response [18,19], little is known regarding the role of lncRNAs in lung fibroblast function or IPF.

In this report, we have demonstrated differences in the functional responses between fibroblasts derived from control and IPF lungs. These are reflected by changes in H3K4me1, a histone epigenetic marker of primed genes and enhancers [26,27] and up-regulation of two lincRNAs, LINC00960 and LINC01140 in IPF fibroblasts. Functional analysis has shown that the both LINC00960 and LIN01140 are required for proliferation and that LINC01140 is a negative regulator of the inflammatory response. Given that LINC01140 is upregulated in both IPF fibroblasts and lung biopsies, our data suggest that LINC1140 mediates the reduced inflammatory response in IPF fibroblasts.

2.3. Methods

Source and fibroblast cell culture

Control (age = 50 ± 3y; 3 male and 2 females) and IPF fibroblasts (age = 62 ± 1y; 3 male and 2 females) were obtained from Professor Carol Ferghali-Bostwick (Medical University of South Carolina, USA) and the Coriell Institute of Medical Research (Camden, New Jersey, USA). Approval was obtained from the Medical Board of the Medical University of South Carolina and the Coriell Institute of Medical Research with patients proving material with informed consent. All methods were performed in accordance with the relevant guidelines and regulations. Neither the control or IPF patients had a history of smoking. Isolation of lung fibroblasts was initiated using explants of minced lung tissue. Fibroblasts were cultured in DMEM (high glucose, pyruvate) growth media (11995-073, ThermoFisher) supplemented with 10% (v/v) FBS (Fetal Bovine Serum) (11550356, ThermoFisher), 1% (v/v) Penicillin-Streptomycin (11548876, ThermoFisher) and 0.1% (v/v) Fungizone (15290-018, ThermoFisher). All cultures were maintained in a 37^{o}C, 5% (v/v) CO_2 humidified incubator. Upon reaching approximately 80-90% confluency cells were washed in sterile 1x PBS (Phosphate Buffered Saline) (P5493, Sigma-Aldrich) followed by treatment with StemPro® Accutase® cell detachment solution (11599686, ThermoFisher). All experiments were performed using cells plated at passage 6 to 7.

Plating and treatment of fibroblasts for pharmacological studies

1 x 10^4 cells were plated in 96 cell culture wells on day 1 and allowed to adhere overnight. On day 2, cells were serum-deprived by reducing FBS to 0.1% in 200µl of starvation media. Fibroblasts were then treated with the required concentration of TGF-β1 (recombinant human, expressed in Chinese hamster ovary cell line, R&D systems, 240-B-002/CF) and IL-1β (recombinant, expressed in *E. coli*, Sigma-Aldrich, I9401-5UG) and incubated for the indicated time before supernatants were collected for protein quantification assessment.

Preparation and treatment of fibroblasts used in microarray analysis

5 x 10^5 cells of control and IPF lung fibroblasts were seeded in 25 cm^2 cell culture flasks (Nunc EasyFlasks, Thermo Fisher Scientific) and medium was replaced until the cells reach near-confluency state. Their growth medium was then replaced with 3ml starvation medium (0.1% FBS) and left overnight. The following day, the cells were treated with and without 3 ng/ml TGF-β1 for 24 hours (hrs) before all supernatants were collected and cells were lysed.

Cell Proliferation

Lung fibroblast proliferation was evaluated by measuring cell viability with the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich). Cells (5000) were seeded in a 96-well plate and incubated overnight in 100 µl growth media (10% FBS). The following day, the cells were serum-deprived and treated with the indicated concentration of PDGF-AB (recombinant human, *E. coli* derived, R&D systems, 222-AB-010) and then incubated for the indicated times. Before the end of each assay, 10 µl of the CCK-8 solution was added into a final volume of 100 µl cultured media and incubated for 2 hrs before absorbance was measured using a microplate reader (Fluostar Optima, BMG Labtech). The absorbance wavelength was measured at 450 nm and 600 nm which was then subtracted during data analysis.

Transfection with LNA antisense

On the day of transfection, 5 µL of HiPerFect (Qiagen) was mixed with 200 µL of growth media without antibiotics, serum or antifungals to prepare the

transfection mix. LNA GapmeRs were added to 200 µL of the transfection mix at a final concentration of 30 nM, placed in 12-well plates and incubated for minimum 10 mins at room temperature. Fibroblasts were then seeded at a density of $5x10^5$ cells per well in 200 µL of growth media and incubated with the transfection mixes at 37^oC, 5% (v/v) CO_2 overnight. The next day, 800 µL of media (0.1% FBS) was added to the wells to dilute out the lipid-LNA complexes and reduce the toxicity of the reaction. The cells were stimulated with either 3 ng/ml of TGF-β1 or 3 ng/ml of IL-1β and incubated for 24 hrs before harvesting for RNA extraction and ELISA (Enzyme-linked immunosorbent assay) analysis. The same transfection protocol was followed for the cell viability assays (CCK-8 kit) with or without 100 ng/ml of PDGF-AB stimulation for 72 hrs, however 96 well plates were used and the reaction volume and reagents were reduced accordingly. LNA GapmeR sequences: Negative Control LNA1 - TCATACTATATGACAG; Negative Control LNA2 - GACGGTAAGTAGGCGA; LINC00960 LNA1 – GGCGTGAGAGTAAAGC; LINC00960 LNA2 – GTGCTTAGGCTTAGAG; LINC01140 LNA1 – TTTAATTGGGCCGTCT; LINC01140 LNA2 – TTGACACGGCTGACTT.

Measurement of IL-6 and PAI-1 release

Supernatants of cultured lung fibroblasts were collected and used to assess secretion of IL-6 and PAI-1, using the DuoSet ELISA (DY206 and DY1786) Development System Kits (R&D Systems Europe, UK) following the manufacturer's instructions. Samples and standard curve samples were diluted as appropriate in reagent diluent. Absorbance was measured at 450nm with wavelength correction at 570nm using a microplate reader (Fluostar Optima, BMG Labtech).

RNA isolation and quality control

For all samples, total RNA was extracted using the RNeasy kit (Qiagen), included an on-column DNase treatment (Qiagen), according to the manufacturer's guideline. RNA concentration was determined using the Qubit 2.0 (Life Technologies). RNA quality was measured using the Agilent Bioanalyzer and produced RIN values of >8.0.

Quantitative PCR validation of lncRNA expression

For quantitative PCR (qPCR), cDNA libraries were prepared from total RNA using the High capacity cDNA RT kit (Applied Biosystems, Life Technologies, 4368813). Expression of mRNAs and lncRNAs were determined by qPCR using the SYBR® Green PCR mix (Applied Biosystems; primers were obtained from Sigma-Aldrich and are listed in Supplementary Table 1). For analysis, the $2^{-(\Delta\Delta Ct)}$ method was used to determine relative-quantities of individual mRNAs and lncRNAs which were normalized to 18S ribosomal RNA. qRT-PCR primer sequences: 18S – AAACGGCTACCACATCCAAG (Forward), CCTCCAATGGATCCTCGTTA (Reverse); IL-6 – ACTCACCTCTTCAGAACGAATG (Forward), CCATCTTTGGAAGGTTCAGGTTG (Reverse); LINC00960 – TCCAGGCGTCATAACCAACC (Forward), CGGTGCTTAGGCTTAGAGGG (Reverse); LINC01140 – CATCTCATCGGCATGGACCT (Forward), CAAACTGGACTGACTTTCACCA (Reverse).

Transcriptome analysis of microarray data

The Affymetrix GeneChip™ Command Console Software was used to summarise probe intensity data and to generate a CEL file for each sample. The CEL files were then processed by the Affymetrix Expression Console™ using Robust Multi-chip Analysis (RMA) to generate CHP files (Probe-level summarisation) following the manufacturers manual. The CHP files were then used for Quality Control analysis which generated a full report with the array QC metrics and appropriate algorithm parameters. Using the data of the CHP files, a 3-dimensional Principle Component Analysis (PCA) graph was generated accounting for the majority of variance based on a set of variables PCA1, PCA2 and PCA3 in the original data set. CHP files generated by the Expression Console Software were used in the Transcriptome Analysis Console (TAC) Software to perform statistical analysis and obtain a list of differentially expressed genes. To run the TAC software, a library folder containing the annotation files was required (HTA-2_0.na36.hg19.probeset.csv) and installed. CHP files were imported and separated into different condition groups for analysis using the Gene Level Differential Expression Analysis tool. A master table was generated containing

signal expression levels of all 67528 transcript clusters (genes) covered in the arrays, 44699 protein coding and 22829 non-protein coding. Signal intensity for each transcript cluster was presented as a Bi-weight average signal (log2) value and depending on which two conditions were compared the values of ANOVA p-value, FDR p-value (based on Benjamini-Hochberg Step-Up FDR-controlling Procedure) and gene fold change were adjusted accordingly.

Transcriptome analysis of sequencing data from lung biopsy samples

Previously reported sequencing data obtained from the biospies of 19 control and 20 IPF lungs (GSE92592) [50] were downloaded from Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) using the following command in SRA tools: fastq-dump -I --split-files <file_name>. The paired end reads were aligned to the human reference genome (hg38) using Hisat2 (version 2.0.4) [51,52] using the following command line options: hisat2 -q --dta --rna-strandness FR –x <reference-genone.gtf> -1 <forward_strand.fa> -2 <reverse-strand file.fa> –S <output.sam>. Output SAM files were then sorted and converted to BAM files (samtools sort -@ 8 –o output.bam output.sam) and indexed (samtools index –b output.bam) in Samtools [53]. The profile of gene expression (using the Gencode v27 database) in the BAM files for each samples were determined using Stringtie [54]: stringtie <sample.BAM> -G <GenCodev26.gtf> -o <samples.gtf> -e -A <sample.txt>. Following feature counting: featureCounts -a <reference-genome.gtf> -g gene_name -o counts.txt Control_*.bam IPF_*.bam the differential gene expression was assessed using DeSeq2 and the following R script: curl -s -O http://data.biostarhandbook.com/rnaseq/code/deseq2.r cat simple_counts.txt | Rscript deseq2.r 19x20 > results_deseq2.txt.

Chromatin immunoprecipitation, sequencing and analysis of H3K4me1

Chromatin immunoprecipitation (ChIP) using a H3K4me1 antibody (Diagenode, C15410037) and the iDeal ChIP-seq kit for Histones kit (Diagenode, C01010051) was performed on the 5 control and 5 IPF fibroblast samples. In addition, we performed ChIP on a single control and IPF using an IgG antibody to provide a background control. Paired-end 75bp sequencing data were obtained using the Illumina HiSeq4000 at the Oxford Genomics

Centre at the Wellcome Centre for Human Genetics (funded by Wellcome Trust grant reference 203141/Z/16/Z). FASTQ sequencing data from control (n=5) and IPF (n=5) fibroblasts was aligned to hg38 using Bowtie 2 [55]: bowtie2 -q --very-fast <reference_genome.gtf> -U <file_name.fastq> -S <output_file.sam>. Output SAM files were then sorted and converted to BAM files (samtools sort -@ 8 –o output.bam output.sam), indexed (samtools index –b output.bam) in Samtools [53] and then converted to BigWig format using BamCoverage (which is part of the deepTools suite [56]) using the following command line: bamCoverage -b <input_bam.bam> --normalizeUsingRPKM --binSize 30 --smoothLength 300 -p 10 --extendReads 200 -o <output_file.bw>. Significant ChIPseq peaks (q = <0.1) in each sample were called with MACS2 [57] using the broadpeak options: macs2 callpeak –t <sample> -c <backgrounf_igG> --broad - <output_files> -g hs. The differential expression of H3K4me1 peaks was determined by inputing the individual Bam files (Bowtie2) and BED files (Broadpeaks output - MACS2) for control and IPF samples into Diffbind (version 2.2.1) on Galaxy (at www.usegalaxy.org) [58]. Control 1 was omitted as an outlier following PCA analysis.

Principle Component Analysis and Hierarchical Clustering

The abundance of Gencode v27 defined genes in individual samples was defined as the fragments per kilobase exon per million reads mapped (FPKM) and determined using Stringtie (RNA). PCA and hierarchical clustering on Gencode v27 genes demonstrating an expression >1 FPKM was performed using Genesis (v1.7.7) [59]. Data were log2 transformed following the addition of 1 FPKM. The threshold for reporting gene expression at FPKM >1 is based upon the ability to validate sequencing data using qRT-PCR.

Data Access

The microarray and ChIPSeq sequencing data is available from the gene expression omnibus under *GSEXXXXX and GSEXXXXX.* The RNA data for control and IPF biopsies is available GSE92592.

Supplementary data (provided on USB memory stick).

Statistical Analysis

All statistical analysis and graphs were generated using GraphPad Prism 7 software.

2.4. Results

Our initial aim was to determine whether there were significant differences in the phenotypic responses of lung fibroblasts derived from control lung and IPF patients. We selected high-throughput approaches to measure the time- and concentration dependency of their TGF-β1-induced fibrotic response, PDGF-induced proliferation and IL-1β-stimulated inflammatory response.

Comparison of the TGF-β1-stimulated PAI-1 release from control and IPF lung fibroblasts

TGF-β1-induced activation of lung fibroblasts triggers the expression of PAI-1 (also known as Serpin E1), a protein known as an important regulator of fibrinolysis and wound healing and therefore implicated in the process of fibrosis [28]. To assess potential difference in the fibrotic response in control and IPF fibroblasts we examined the time- and concentration-dependent release of PAI-1 release in response to TGF-β1.

Initial studies showed a time-dependent release of PAI-1 from non-stimulated IPF cells, which reached significance at 48 hrs and 72 hrs (Figure 1A/B). However, comparison between control (4.4 ± 2.2 ng/ml) and IPF (5.1 ± 1.4 ng/ml) at 72 hrs showed no significant difference. Exposure to TGF-β1 produced a comparable time dependent increase in PAI-1 release from control and IPF fibroblasts, that was significant at 24 hrs and continued to increase at 48 hrs and 72 hrs (Figure 1C/D). There was no significant difference between the control and IPF fibroblasts at 72 hrs with absolute values of 25.3 ± 2.0 ng/ml and 26.6 ± 5.3 ng/ml, respectively.

To examine the concentration-dependent response, cells were incubated for 72 hrs with 0.01 to 10 ng/ml TGF-β1 and compared with time matched non-stimulated cells. As expected, TGF-β1 induced a concentration-dependent increase in PAI-1 release in both control (Figure 1E) and IPF (Figure 1F) fibroblasts that plateaued at 21.6 ng/ml and 19.0 ng/ml, respectively. The baseline expression in non-stimulated control and IPF fibroblasts was 2.7 ng/ml and 3.4 ng/ml. Comparison of the mean logEC_{50} between the control (pEC_{50} = 0.49 ± 0.05) and IPF (pEC_{50} = 0.85 ± 0.13) individuals showed a significant difference ($p < 0.05$; unpaired t-test). In relation to PAI-1 release, these studies show IPF fibroblasts are more fibrotic since they demonstrate increased sensitivity to TGF-β1 activation.

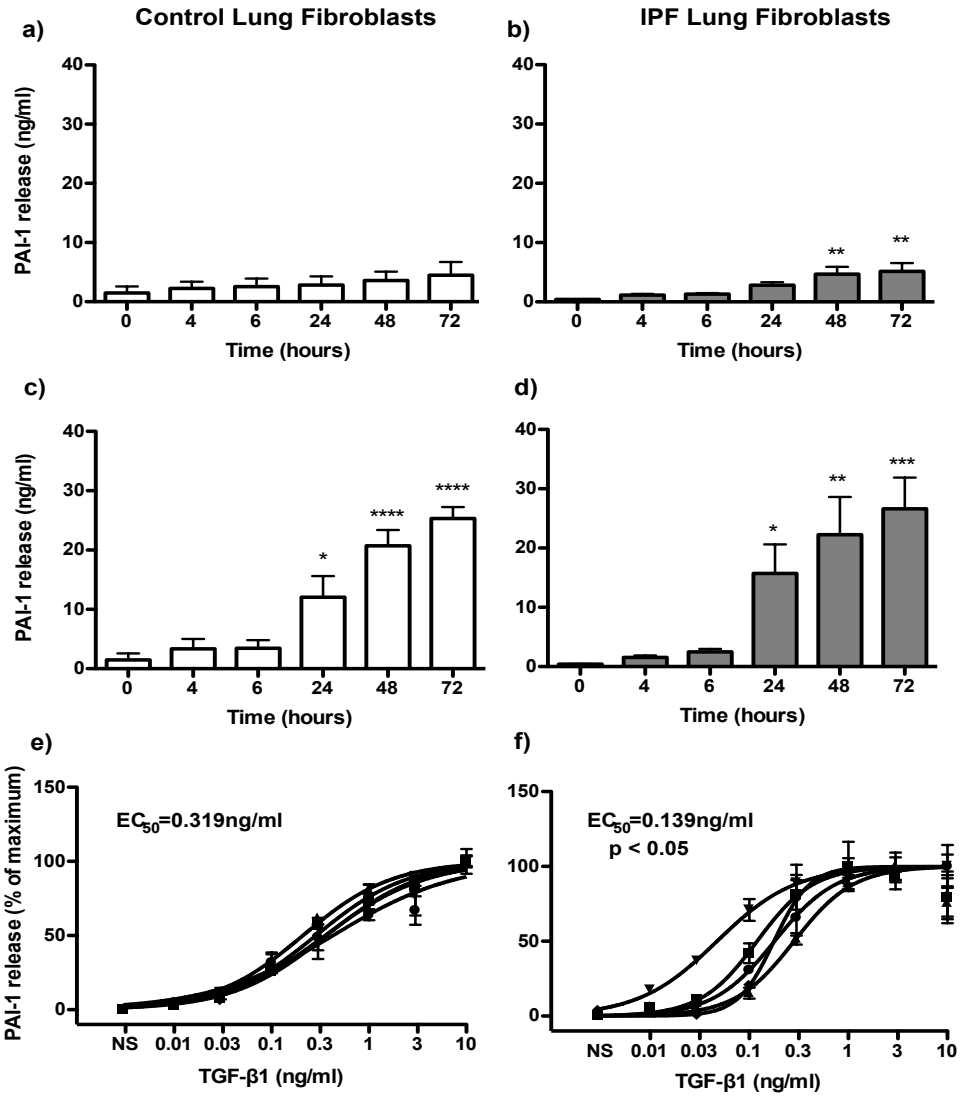


Figure 1. IPF lung fibroblasts showed increased sensitivity to TGF-β1-stimulated PAI-1 release. Time course of PAI-1 release from non-stimulated

(A/B) and TGF-β1-stimulated (C/D) fibroblasts derived from control (white) (A/C) and IPF (grey) (B/D) patients. PAI-1 release from control (E) and IPF (F) fibroblasts at 72 hrs following exposure to the indicated TGF-β1 concentrations. Data represents the mean +/- SEM of five individuals. Statistical significance was performed using 1-way analysis of variance (ANOVA) with a Dunnett's test for time courses (A-D) where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001. The $\log EC_{50}$ for each individual was determined in GraphPad Prism and comparison between control and IPF groups was performed using an unpaired t-test. The EC_{50} was calculated from the mean $\log EC_{50}$ values.

Comparison of PDGF-AB-stimulated proliferation in control and IPF lung fibroblasts

PDGF-AB is a potent mitogen that has been previously shown to stimulate the proliferation of lung fibroblasts [29]. To assess the proliferative response in control and IPF fibroblasts, we investigated the time- and concentration-dependent effect of PDGF-AB on the fibroblast cell number. Initial examination of the proliferation in non-stimulated fibroblasts showed increased proliferation in control versus IPF at 72 hrs (Figure 2A). On top of the baseline increases demonstrated in Figure 2A, exposure to 100 ng/ml PDGF-AB was demonstrated to induce an additional time dependent proliferation in both control and IPF fibroblasts, with an increase 1.8 fold and 2.1 fold at 72 hrs, respectively (Figure 2B/C).

Following exposure to recombinant human PDGF-AB (0.3 – 300 ng/ml), we observed a concentration-dependent increase in proliferation at 72 hrs, although we observed wide variation in the response of IPF fibroblasts (Figure 2E) compared with controls (Figure 2D). Comparison of the mean $\log EC_{50}$ values showed no significant difference between control and IPF fibroblasts. These results indicated that there was a significant increase in proliferation in non-stimulated control fibroblasts versus IPF, there was no difference in the PDGF-stimulated responses.

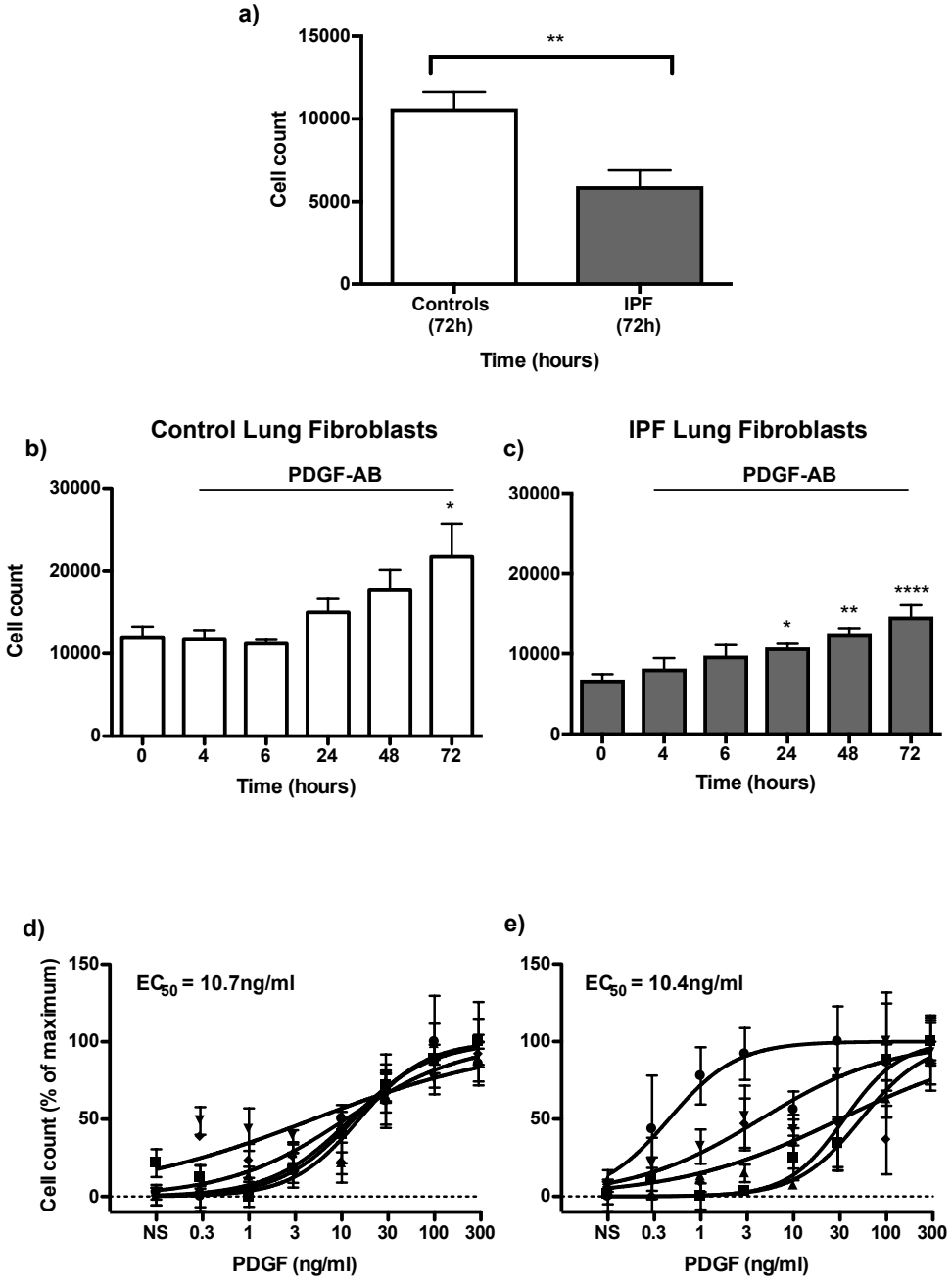


Figure 2. Non-stimulated IPF lung fibroblasts show a reduced proliferative response. Proliferation in non-stimulated control (white) and IPF fibroblasts (grey) was measured 72 hrs using cell count (A). Time course of proliferation in PDGF-stimulated (from control (B) and IPF (C) patients). Proliferation in control (D) and IPF (E) fibroblasts at 72 hrs following exposure to the indicated PDGF concentrations. Data represents the mean +/- SEM of five individuals. Statistical significance was performed using 1-way analysis of variance (ANOVA) with a Dunnett's test for time courses (A-C) where * = *p*

<0.05, ** = p <0.01 and **** = p <0.0001. The logEC_{50} for each individual was determined in GraphPad Prism and comparison between control and IPF groups was performed using an unpaired t-test. The EC_{50} was calculated from the mean logEC_{50} values.

Comparison of IL-1β-stimulated IL-6 release in control and IPF lung fibroblasts

The pro-inflammatory cytokine IL-1β has been shown to potently induce IL-6 release from various cell types including fibroblasts [30,31]. To assess potential differences in the inflammatory response, we examined the time- and concentration-dependent IL-1β induced IL-6 release from control and IPF lung fibroblasts.

Interestingly, we observed a time dependent IL-6 release in non-stimulated fibroblasts, which was significantly increased in control (67.1 ± 19.8 pg/ml) versus IPF (3.7 ± 0.9 pg/ml) fibroblasts at 72 hrs (p = 0.018: unpaired t-test) (Figure 3A/B). Exposure to 3 ng/ml IL-1β induced a comparable time-dependent release of IL-6 from control and IPF fibroblasts, which was initially detected at 4 hrs and continued to increase over the 72 hrs period, with a maximum of 723 ± 134 pg/ml and 483 ± 85 pg/ml, respectively (Figure 3C/D). There was no significant difference in the maximal values between control and IPF at 72 hrs (Figure 3C/D).

To examine the concentration-dependency, cultured cells were incubated for 72 hrs with increasing concentrations of IL-1β ranging from 0.03 to 30 ng/ml (Figure 3E/F). Comparison of the logEC_{50} values showed a significant reduction (p = 0.0234) between control (pEC_{50} = 0.99 ± 0.19) and IPF (pEC_{50} = 0.25 ± 0.17) fibroblasts. Based upon IL-6 release, this data would indicate that IPF fibroblasts are less inflammatory than control cells as they demonstrated both reduced sensitivity to IL-1β and a lower basal release of IL-6.

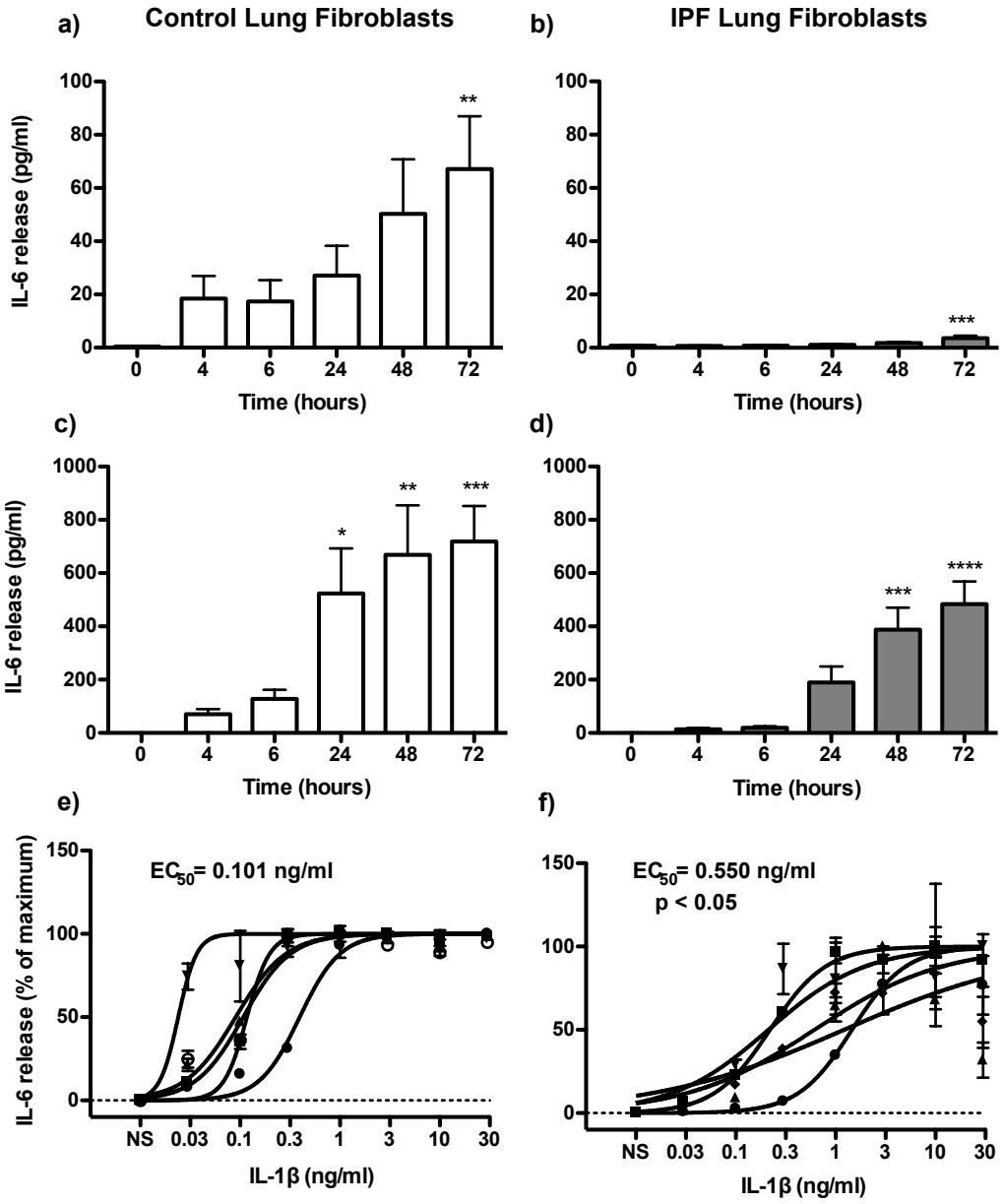
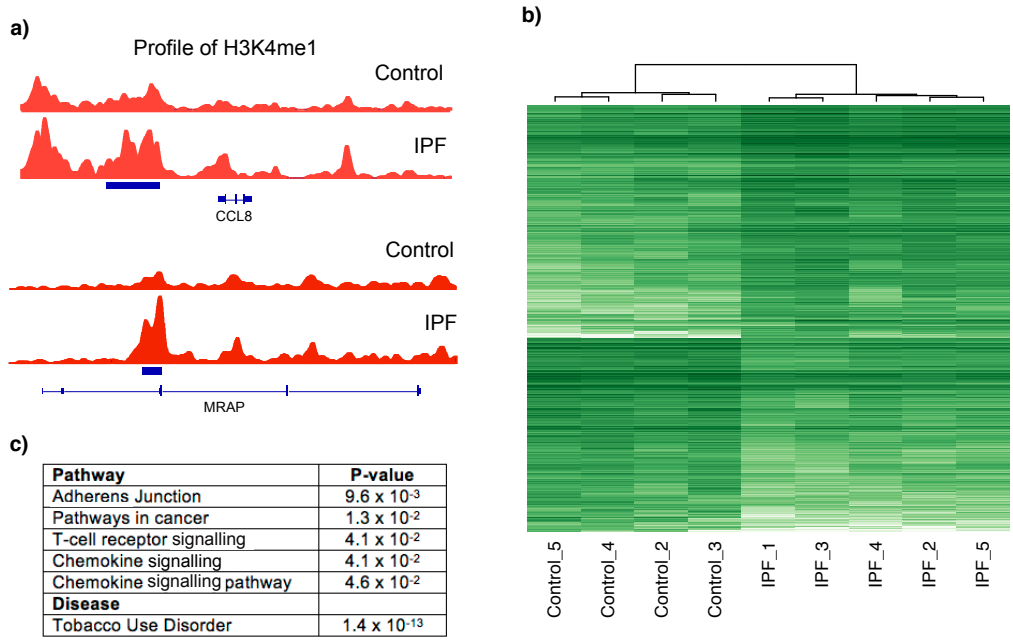


Figure 3. IPF lung fibroblasts show a reduced inflammatory response. Time course of IL-6 release from non-stimulated (A/B) and IL-1β-stimulated (C/D) fibroblasts derived from control (white) (A/C) and IPF (grey) (B/D) patients. IL-6 release from control (E) and IPF (F) fibroblasts at 72 hrs following exposure to the indicated IL-1β concentrations. Data represents the mean +/- SEM of five individuals. Statistical significance was performed using 1-way analysis of variance (ANOVA) with a Dunnett's test for time courses (A-D) where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001. The logEC_{50} for each individual was determined in GraphPad Prism and comparison between control and IPF groups was performed using an unpaired t-test. The EC_{50} was calculated from the mean logEC_{50} values.

Phenotypic differences between control and IPF fibroblasts are reflected at the epigenetic level

Having demonstrated differentially functional responses between control and IPF fibroblasts, we examined whether these differences were reflected at the epigenetic level. To this end we examined the histone modification H3K4me1, a marker that has previously been associated with primed promoter and enhancer regions [26,27]. Comparison between non-stimulated control and IPF fibroblasts identified 462 regions of differential expression (Supplemental Table 1). As examples, we have included the profiles of the H3K4me1 peaks associated with CCL8 and MRAP (Figure 4A). Unsupervised hierarchical clustering showed a clear difference (separation) of the control and IPF samples (Figure 4B) indicating that the phenotypic differences are indeed reflected at the epigenetic level. Pathway analysis of the genes in which these regions overlapped or which were closest (Supplemental Table 1) showed that these were associated with tight junctions, cancer and inflammation (Figure 4C). Interestingly, these genes were also strongly associated with tobacco user disorder (1.4×10^{-13}) despite no history of smoking in either the control or IPF patients.



c)

Pathway	P-value
Adherens Junction	9.6×10^{-3}
Pathways in cancer	1.3×10^{-2}
T-cell receptor signalling	4.1×10^{-2}
Chemokine signalling	4.1×10^{-2}
Chemokine signalling pathway	4.6×10^{-2}
Disease	
Tobacco Use Disorder	1.4×10^{-13}

Figure 4. Differential expression of the H3K4me1 epigenetic mark between control and IPF fibroblasts. ChIP sequencing was employed to examine the differential expression of H3K4me1, a marker of primed promoter and enhancer regions. (A) examples of the H3K4me1 regions associated with CCL8 and MRAP, (B) unsupervised hierarchical clustering was calculated within the DiffBind programme and (C) pathways analysis of H3K4me1 associated genes was undertaken using DAVID.

Differential long non-coding RNA expression between control and IPF fibroblasts

Having demonstrated differentially functional responses between control and IPF fibroblasts, we wondered whether these might be related to long non-coding RNAs (lncRNAs). To this end, we compared the expression profile in control and IPF lung fibroblasts in non-stimulated cells and those exposed to 3 ng/ml TGF-β1 for 24 hrs, using the Affymetrix GeneChip™ Human Transcriptome Arrays 2.0 (Supplemental Table 2). TGF-β1 exposure resulted in widespread and shared changes in gene expression in both control (1331 genes including 10 lincRNAs and 14 antisense: Supplemental Table 3) and IPF fibroblasts (1424 genes including 15 lincRNAs and 13 antisense:

Supplemental Table 4) (Figure 5A), q <0.05. As might be expected, pathway analysis (DAVID Bioinformatics) showed that the highest hit for the up-regulated genes were extracellular matrix (9.9×10^{-15}). Amongst this group, PAI-1 (Serpin E1) gave one of the highest fold changes in both the control (44-fold) and IPF fibroblasts (31-fold). Comparison of the lists of genes that were changed following TGF-β1 exposure identified only 77 that were differentially expressed between control and IPF, which includes a single lincRNA (LOC100507516) whose expression was reduced in IPF (Supplemental Table 5).

To identify those lncRNAs that might regulate the fibrotic, proliferative and inflammatory response, we compared the profile of gene expression in non-stimulated fibroblasts. This identified differential expression of 104 genes including 2 lincRNAs (LINC00960 and LINC01140) that were increased in IPF (Supplemental Table 6), p <0.05. Interestingly, there was also a general down-regulation of small nucleolar RNAs (snoRNAs), that are commonly associated with splicing, as well as changes in a number of inflammatory genes including CXCL8 down-regulation and CXCL11 up-regulation (Supplemental Table 6).
In subsequent functional studies, we focused upon the role of the lincRNAs, LINC00960 and LINC01140. Initial qRT-PCR analysis confirmed their upregulation in IPF fibroblasts (Figure 5B) whilst analysis of ENCODE data from human fibroblasts demonstrated their expression using RNA sequencing data and two epigenetic markers of active promoters (H3K4me3) and transcription (H3K27ac) (Figure 5C). To ascertain whether these are also upregulated in IPF lung *in situ*, we analysed RNA sequencing data of biopsy samples obtained from control (n=19) and IPF (n=20) lungs [32] (Supplemental Data 8) and showed significant upregulation of LINC01140 but not LINC00960 (Figure 5D).

a)

	mRNA	lincRNA	antisense	pseudogenes	snoRNA	miRNA	Others	Total
Non-stimulated (control vs IPF)	90	2	2	0	7	0	3	104
TGF-stimulated (control vs IPF)	50	1	0	1	19	1	5	77
Control (TGF stimulated)	1265	10	14	17	3	9	13	1331
IPF (TGF stimulated)	1356	15	13	18	0	5	17	1424

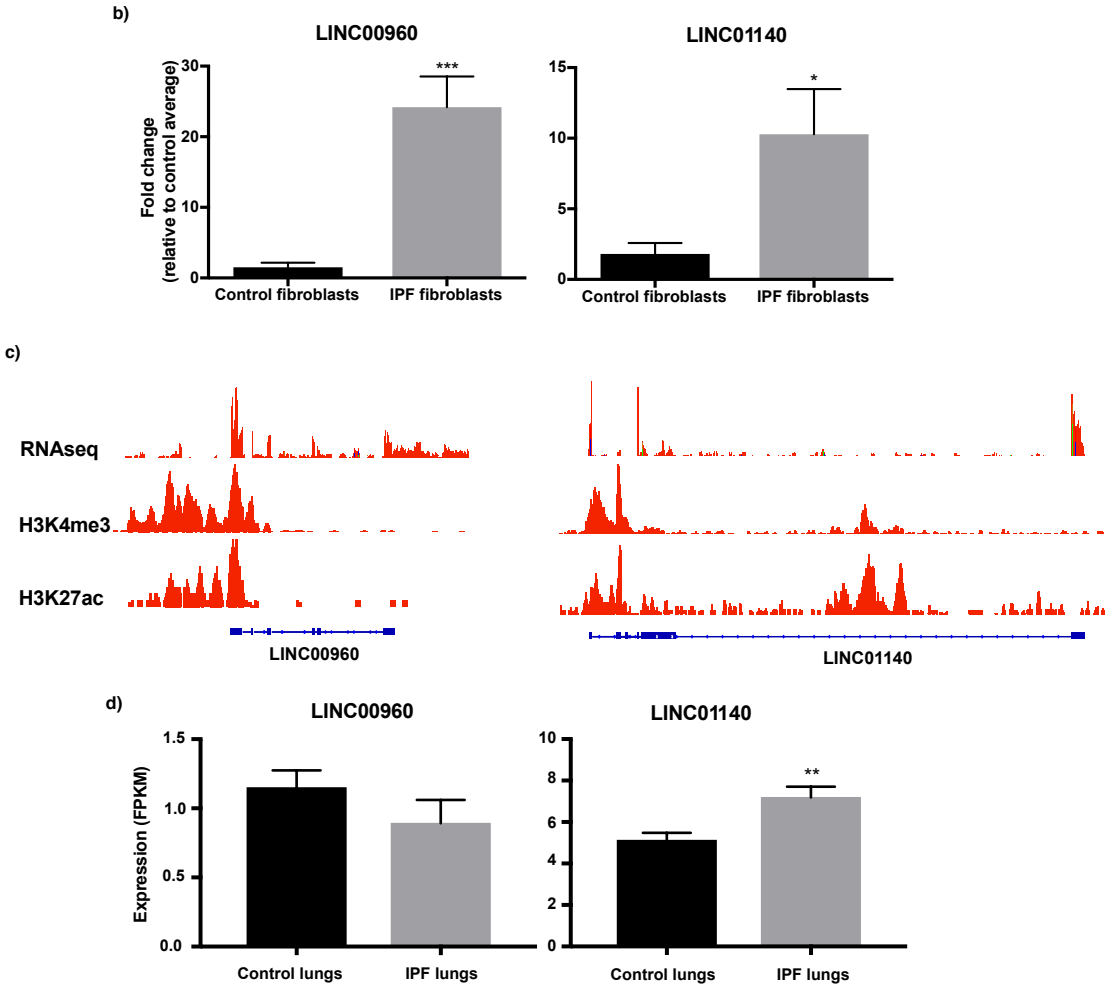


Figure 5. Transcriptome analysis shows differential expression of long intergenic RNA between control and IPF fibroblasts. (A) The differential expression of various classes of genes was examined in 5 control and 5 IPF fibroblasts samples in the presence and absence of TGF-β1 stimulated at 24 hrs, q< 0.05. (B) The differential expression of the two lincRNAs, LINC00960 and LINC01140 was confirmed by qRT-PCR (n=5). (C) LINC00960 and LINC01140 expression was confirmed by comparison with RNA sequencing data and the epigenetic marks associated with H3K4me3 (active promoters) and H3K27ac (active transcription). (D) Expression of LINC00960 and LINC01140 in the lung biopsies of control (n=19) and IPF patients (n=20).

Data in B and D are the mean +/- SEM and statistical significance was performed using an unpaired t-test where * = p <0.05, ** = p <0.01 and *** = p <0.001.

Long intergenic non-coding RNAs and the regulation TGF-β1-stimulated PAI-1 release from control and IPF lung fibroblasts

To investigate the function of LINC00960 and LINC01140, we identified 2 locked nucleic acid based (LNA) antisense sequences against each lincRNA that produced 50-85% knockdown following overnight transfection into fibroblasts and stimulation with TGF-β1 for 24 hrs (Figure 6A). Following exposure to TGF-β1, we observed a significant increase in PAI-1 release from both control and IPF fibroblasts (Figure 6B), although the magnitude of this response was smaller than that observed in non-transfected cells (Figure 1). Knockdown of LINC00960 had no effect upon PAI-1 release from both control and IPF fibroblasts. Although one LNA antisense against LINC01140 caused a significant reduction in PAI-1 release from control fibroblasts (Figure 6B), taken as a whole, it appears that LINC01140 also does not regulate PAI-1 release. Two scrambled negative LNA controls had no effect on either lincRNA expression or PAI-1 release. These results indicate that neither LINC00960 nor LINC01140 are required for PAI-1 regulation in control and IPF fibroblasts.

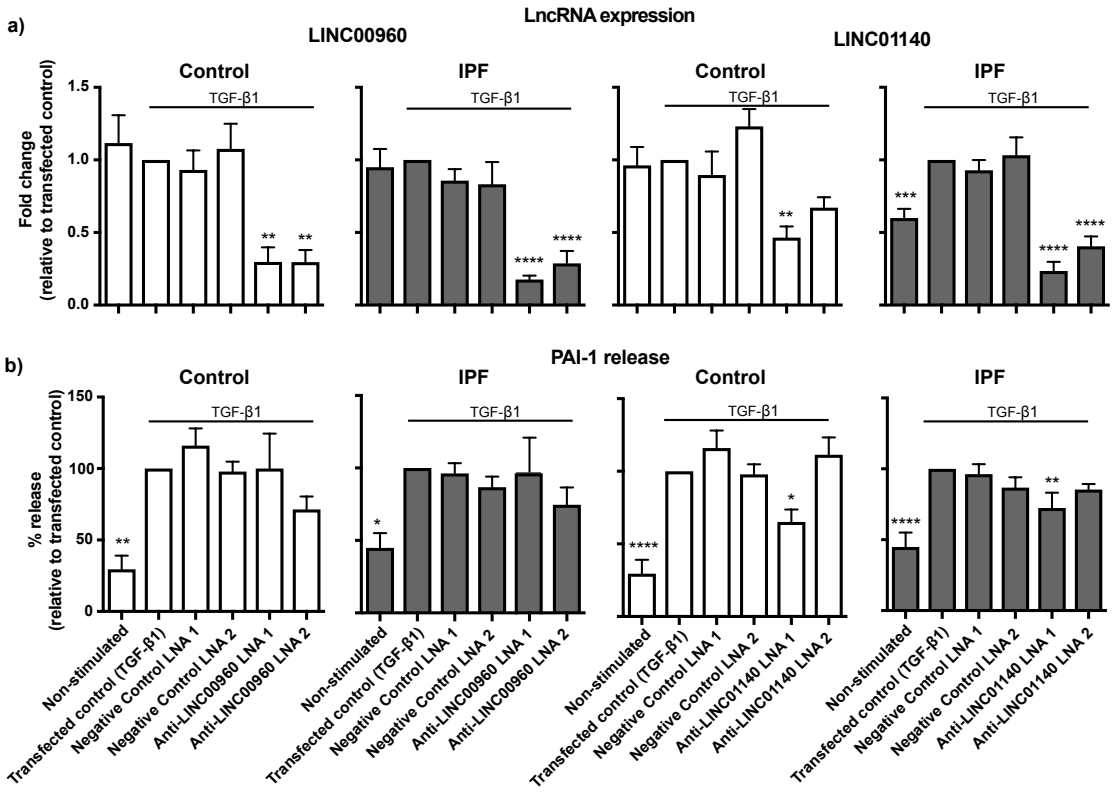


Figure 6. LincRNAs and the regulation of TGF-β1-stimulated PAI-1 release. Control and IPF fibroblasts were transfected with LNA antisense sequences against LINC00960, LINC01140 or scrambled controls overnight. Cell were then stimulated with TGF-β1 for 24 hrs prior to (A) isolation of mRNA and measurement of LINC00960 or LINC01140 by qRT-PCR or (B) measurement of supernatant PAI-1 by ELISA. Data represents the mean +/- SEM of five control or IPF individuals. Statistical significance was performed using the repeat measures 1-way analysis of variance (ANOVA) with a Dunnett's test where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001.

Long intergenic non-coding RNAs and the regulation of PDGF-stimulated proliferation in lung fibroblasts

Following overnight transfection with LNA antisense to LINC00960, LINC01140 and the 2 negative controls, we observed no effect upon cell count, indicating that this procedure had no immediate action upon cell viability (Figure 7A). Following 72 hrs culture, knockdown of LINC00960 and LINC01140 caused a significant reduction in proliferation in both non-stimulated (Figure 7B) and PDGF-stimulated (Figure 7C), which was seen in both control and IPF fibroblasts. Generally, no reduction was observed with the negative LNA controls. These results indicate that LINC00960 and LINC01140 are positive regulators of fibroblast proliferation.

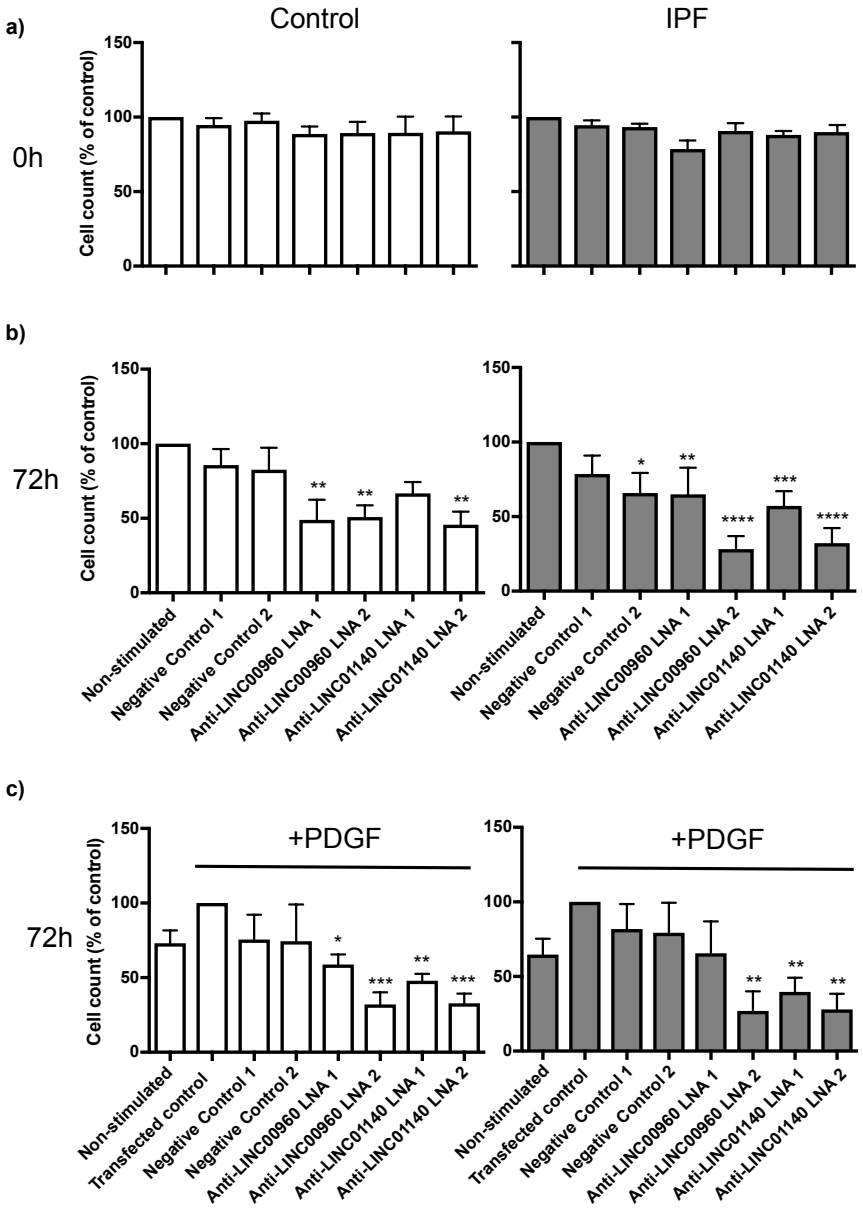


Figure 7. LincRNAs and the regulation of the PDGF-AB-stimulated proliferation. Control and IPF fibroblasts were transfected with LNA antisense sequences against LINC00960, LINC01140 or scrambled controls overnight. The cell number was determined at 0 hrs (A) or in non-stimulated (B) and PDGF-stimulated (C) samples at 72 hrs. Data represents the mean +/- SEM of five control or IPF individuals. Statistical significance was performed using the repeat measures 1-way analysis of variance (ANOVA) with a Dunnett's test where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001.

Long intergenic non-coding RNAs and the regulation of IL-1β-stimulated IL-6 release in control and IPF lung fibroblasts

We once again demonstrated LNA mediated knockdown of LINC00960 and LINC01140 in both control and IPF fibroblasts following overnight transfection and 24 hrs exposure to IL-1β (Figure 8A). LINC000960 knockdown had no effect upon either IL-1β-induced IL-6 expression (Figure 8B) or IL-6 release (Figure 8C) from either control or IPF fibroblasts. In contrast, LINC01140 knockdown in IPF fibroblasts resulted in a ~ 4-7 fold increase in IL-6 mRNA expression (Figure 8B) and ~ 2-3 fold increase in IL-6 release (Figure 8C) although the variability between patients meant that this was only significant with LNA1. A much smaller ~ 2 fold increase in IL-6 mRNA and protein was also seen with LNA2 against LINC01140 in control fibroblasts (Figure 8B/C). These observations indicate that LINC01140 is a negative regulator of the IL-1β-stimulated IL-6 release, particularly in IPF fibroblasts. Interestingly, the increased expression of LINC01140 in IPF fibroblasts might explain their reduced response to IL-1β compared to control fibroblasts (Figure 3).

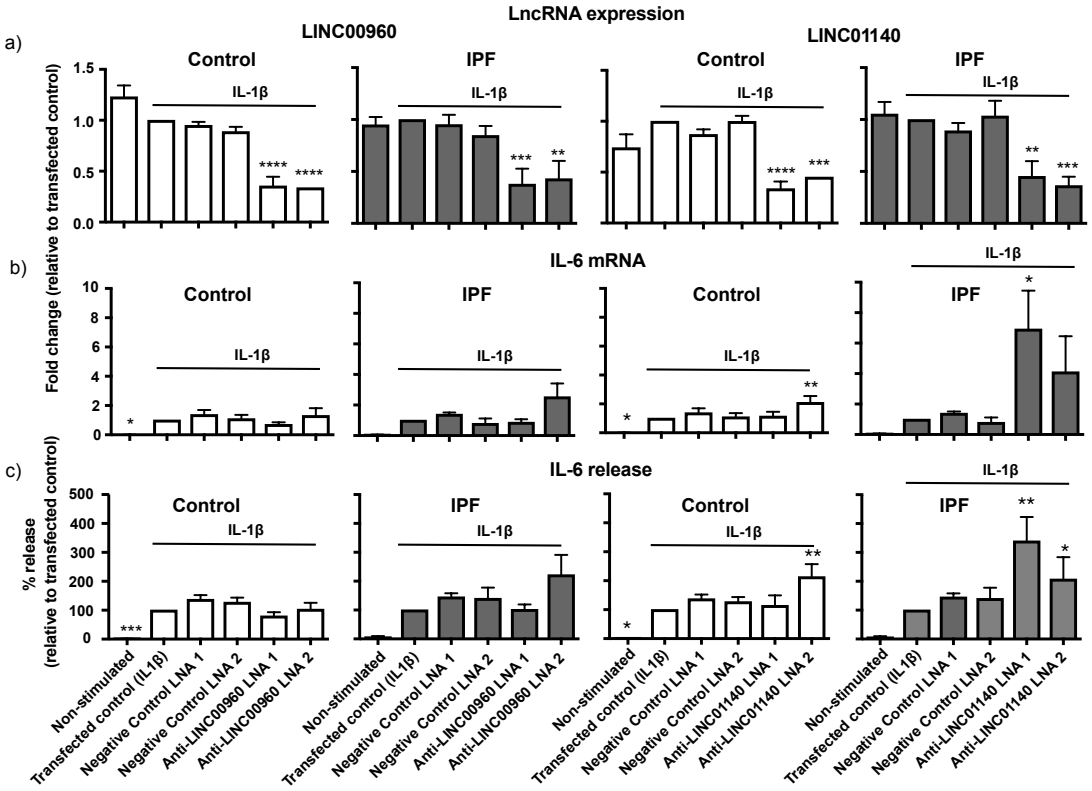


Figure 8. LincRNAs and the regulation of the IL-1β-stimulated IL-6 release. Control and IPF fibroblasts were transfected with LNA antisense sequences against LINC00960, LINC01140 or scrambled control overnight. Cell were then stimulated with IL-1β for 24 hrs prior to isolation of RNA and measurement of LINC00960 or LINC01140 (A) and IL-6 (B) by qRT-PCR or (C) measure of released IL-6 by ELISA (mean raw values: 100% control fibroblasts, 13633 pg/ml; 100% IPF fibroblasts, 5711 pg/ml). Data represents the mean +/- SEM of five control or IPF individuals. Statistical significance was performed using the repeat measures 1-way analysis of variance (ANOVA) with a Dunnett's test where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001.

2.5. Discussion

In this report, we have for the first time investigated the role of lincRNAs in the regulation of lung fibroblast function and whether changes in their expression might be involved in the development of IPF. In our initial studies, we employed high throughput assays to undertake detailed examination of the time and concentration responses of control and IPF fibroblasts, examining phenotypes associated with IPF [1,3]. These included TGF-β1-stimuated PAI-1 release as a marker of the fibrotic response [33,34], PDGF-induced changes in cell numbers as a model of proliferation [35,36] and IL-1β-stimulated IL-6 release as a marker of inflammation [31].

As previously reported when using collagen release as a measure of fibrosis, we observed no difference in the magnitude of PAI-1 release from control and IPF fibroblast in the absence and presence of a maximally effective TGF-β1 concentration [13]. In contrast, the concentration response curves showed a significant leftward shift in IPF fibroblasts, indicating that these had increased sensitivity to TGF-β1.

In the absence of PDGF, we observed a reduction in the proliferation of IPF fibroblasts. Following exposure to PDGF, the small increases and the variability of the response between individual fibroblasts samples, meant there was no significant difference in the magnitude or concentration dependency of the proliferation. This variability in the PDGF-induced proliferative response had previously been observed by Jordana *et al* [10].

In general, IL-1β is considered to be a potent pro-inflammatory cytokine that can induce the release of multiple pro-inflammatory mediators, including IL-6, following the activation of the transcription factor, nuclear factor-kappaB (NF-kB)[37]. Unlike TGF-β1 and PDGF, the role of IL-1β in the development of IPF is yet to be established although there is a report of showing increased levels in bronchoalveolar lavage [38]. In addition, there are conflicting reports as to whether IL-1β elicits profibrotic or antifibrotic activities [39,40]. IL-1β was

previously shown to drive IL-6 expression in orbital and synovial fibroblasts *in vitro* [30], although nothing is known regarding its effect on lung fibroblasts. As anticipated, exposure to IL-1β induced high levels of IL-6 cytokine release in both control and IPF lung fibroblasts in a concentration-dependent manner. Comparison of the magnitude and the concentration response indicated that IPF fibroblasts were less inflammatory than controls. This had also been confirmed during a recent meta-analysis of four previous microarrays studies in control and IPF fibroblasts which showed repression of inflammation and immune pathways [41]. Interestingly, this reduced inflammation may explain the lack of efficacy observed when corticosteroids and immunosuppressants have been examined as therapeutic options for the treatment of IPF [1,3].

Having demonstrated significant differences in fibrotic and inflammatory responses between control and IPF fibroblasts, we then proceeded to show that this was also reflected at the epigenetic level. To date, the only genome wide epigenetic studies in IPF have examined the changes in the profile of DNA methylation [14,15], with none having examined histone modifications. We therefore measured the distribution of H3K4me1, a marker for priming at promoter and enhancer regions that is thought to work through the recruitment of multiple chromatin-remodeling complexes [26,27,42]. Significantly, we identified multiple regions demonstrating differential expression of H3K4me1, with unsupervised hierarchical clustering showing a clear separation between control and IPF fibroblasts. Although there have been a number of reports showing changes is histone marks localised to specific genes implicated in IPF [16], this is the first evidence to show genome-wide changes in histone modifications and that targeting the acetylation of H3K4 and other histones might provide a novel therapeutic strategy. Indeed, this latter contention is supported by studies into Brd4, a bromodomain containing protein that can bind to acetylated histones and act as a scaffold to attract components of the transcriptional machinery. Thus, inhibitors of Brd4 have been shown to attenuate migration, proliferation and IL6 release in isolated fibroblasts and inhibit fibrosis in a bleomycin-induced model of flung fibrosis [43,44].

At the present time, little is known regarding the role of lincRNA either in the regulation of fibroblast function or whether changes in their expression are associated with the phenotypic changes associated with IPF. The exception is studies showing that H19 [45,46] and pulmonary fibrosis associated lncRNA (PFAL) [47] contribute to lung fibrosis by acting as sponges (and therefore inhibitors) for miRNAs. Using microarray analysis and qRT-PCR, we identified 2 lincRNAs, LINC00960 and LINC01140 that were up-regulated in IPF compared to control fibroblasts. Given that isolated and cultured fibroblasts might not reflect the situation in the whole lung, we were able to analyse historical RNA sequencing data obtained from lung biopsies [32] and confirm up-regulation of LINC01140 but not LINC00960. Knockdown studies indicated that neither appeared to regulate TGF-β1-induced PAI-1, although it is not possible to eliminate the possibility that these lincRNAs might regulate another aspect of the fibrotic response. In contrast both LINC00960 and LINC01140 were positive regulators of proliferation whilst LINC01140 was a negative regulator of IL-1β-induced IL-6 release. In the latter case, there was a greater elevation in IL-6 release from IPF fibroblasts following LINC001140 knockdown, possibly reflecting the increased expression of LINC01140 in IPF versus control fibroblasts. This increased LINC01140 levels in IPF fibroblast might also explain the absence of IL-6 release in non-stimulated IPF fibroblasts compared with controls and the shift in the concentration response curve in response to IL-1β exposure. Although the mechanism of action is yet to be determined, negative regulation of the inflammatory response is commonly observed with both lncRNAs and miRNAs including interleukin-7-antisense (IL7-AS) [48] and miR-146a [49].

Overall, this report is the first to demonstrate that phenotypic differences between control and IPF fibroblasts are associated with genome-wide changes in histone modifications and increased expression of the lincRNAs, LINC00960 and LINC01140. Significantly, we also demonstrate that these lincRNAs can regulate fibroblast proliferation and inflammation whilst changes in LINC01140 expression might mediate the reduced inflammatory in IPF fibroblasts.

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3. Chapter 3 – Long non-coding RNAs are central regulators of the IL-1β-induced inflammatory response in human lung fibroblasts

Statement of Authorship Form

This declaration concerns the article entitled:

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Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.							
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Long non-coding RNAs are central regulators of the IL-1β-induced inflammatory response in human lung fibroblasts

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3.1. Abstract

There is accumulating evidence to indicate that long non-coding RNAs (lncRNAs) are important regulators of the inflammatory response. In this report, we have employed next generation sequencing to identify 14 lncRNAs that are differentially expressed in human lung fibroblasts following the induction of inflammation using interleukin-1β (IL-1β). Knockdown of the two most highly expressed lncRNAs, IL7AS and MIR3142HG, showed that IL7AS negatively regulated IL-6 release whilst MIR3142HG was required for IL-8 and CCL2 release. Parallel studies in fibroblasts derived from patients with idiopathic pulmonary fibrosis showed similar increases in IL7AS levels, that also negatively regulate IL-6 release. In contrast, IL-1β-induced MIR3142HG expression was reduced by 8-fold in IPF fibroblasts, with the consequence being that MIR3142 knockdown showed no effect upon IL-8 and CCL2 release. In summary, we have catalogued those lncRNAs that are differential expression following IL-1β-activation of human lung fibroblasts and shown that these regulate the inflammatory response.

3.2. Background

Interleukin-1β (IL-1β) is a potent pro-inflammatory mediator that is produced following activation of one of the multiple inflammasome multi-protein complexes. One of the best characterised is the nucleotide-binding oligomerization domain-like receptor (NLR) family, pyrin domain-containing 3 (NLRP3) inflammation, whose activation and subsequent release of IL-1β, has been demonstrated in chronic obstructive pulmonary disease, severe asthma and respiratory infections (1). Idiopathic pulmonary fibrosis (IPF) is a chronic disease characterised by scar tissue accumulation in the lungs leading to impaired gas exchange and restricted ventilation (2-4). The underlying causes of the disease are still unclear, although persistent epithelial injury and/or exposure to pathogens is thought to drive an exaggerated wound healing response from fibroblasts, that contributes

towards the development and progression of IPF (2,4). In the case of this respiratory disease, the role of IL-1β is yet to be established although there is a report of showing increased levels in bronchoalveolar lavage (5). In addition, there are conflicting reports as to whether IL-1β elicits pro-fibrotic or anti-fibrotic activities (6,7). IL-1β was previously shown to drive IL-6 expression in orbital and synovial fibroblasts *in vitro* (8), although nothing is known regarding its effect on lung fibroblasts.

Non-coding RNAs (ncRNAs) are broadly classified as either short ncRNAs (< 200 nucleotides) or long ncRNAs (> 200 nucleotides). The microRNA (miRNA) family of short ncRNAs are the best characterised and known to induce mRNA degradation and/or suppress mRNA translation via RNA interference pathway (9). There is now a considerable body of evidence to indicate that miRNAs are central regulators of the immune response (10). In particular, induction of miR-146a and miR-155 have been shown to be regulators of the inflammatory response in multiple cells types (11,12). In contrast, much less is known about the function and mechanism of action of lncRNAs which are commonly grouped into long intergenic ncRNA (lincRNA) (located between protein coding genes), antisense (whose transcription overlaps protein coding genes on the opposite strand) and pseudogenes (non-translated versions of protein-coding genes) (13,14). However, either through interactions with proteins and/or RNA/DNA pairing, there it is now accumulating evidence to indicate that lncRNAs are novel regulators of multiple biological response, including inflammation (15,16). Indeed, studies by ourselves and others have identified a number of lncRNAs that are differentially expressed following activation of innate immunity, which have been shown to regulate the subsequent inflammatory response including PACER (p50-associated COX-2 extragenic RNA) (17), THRIL (TNF and hnRNPL related immunoregulatory lincRNA) (18), lnc-IL7R (19), IL1β-RBT46 (20), lincRNA-COX2 (21,22), lincRNA-EPS (23), lincRNA-Tnfaip3 (24) and IL7AS (25).

At the present time, nothing is known regarding the function of lncRNAs in the fibroblast inflammatory response and whether this is changed in IPF. In this report, we have employed next generation sequencing to examined the

changes in the profile of lncRNA expression following the IL-1β-induced inflammatory response from human lung fibroblasts and determined whether these regulate the inflammatory response in both control and IPF-derived fibroblasts.

3.3. Materials and Methods

Fibroblast source and cell culture

Control (age = 50 ± 3y; 3 male and 2 females) and IPF fibroblasts (age = 62 ± 1y; 3 male and 2 females) were obtained from Professor Carol Ferghali-Bostwick (Medical University of South Carolina, USA) and the Coriell Institute of Medical Research (Camden, New Jersey, USA). Approval was obtained from the Medical Board of the Medical University of South Carolina and the Coriell Institute of Medical Research with patients proving material with informed consent. All methods were performed in accordance with the relevant guidelines and regulations. Neither the control or IPF patients had a history of smoking. Isolation of lung fibroblasts was initiated using explants of minced lung tissue. Fibroblasts were cultured in DMEM (high glucose, pyruvate) growth media (11995-073, ThermoFisher) supplemented with 10% (v/v) FBS (Fetal Bovine Serum) (11550356, ThermoFisher), 1% (v/v) Penicillin-Streptomycin (11548876, ThermoFisher) and 0.1% (v/v) Fungizone (15290-018, ThermoFisher). All cultures were maintained in a 37^{o}C, 5% (v/v) CO_2 humidified incubator. Upon reaching approximately 80-90% confluency cells were washed in sterile 1x PBS (Phosphate Buffered Saline) (P5493, Sigma-Aldrich) followed by treatment with StemPro® Accutase® cell detachment solution (11599686, ThermoFisher). All experiments were performed using cells plated at passage 6 to 7.

Preparation and treatment of fibroblasts used in RNA-seq study

Control lung fibroblasts (5 x 10^5 cells) were seeded in 6 cell culture well plates (Corning Costar) on day 1 and left overnight. On day 2, the cells were serum-starved with 2 ml of fresh medium (0.1% FBS) and treated with/without 3 ng/ml IL-1β (recombinant, expressed in *E. coli*, Sigma-Aldrich, I9401-5UG) for 6

hours (hrs) before all supernatants were collected and cells were harvested for RNA extraction.

RNA isolation and quality control

For all samples, total RNA was extracted using the RNeasy kit (Qiagen), included an on-column DNase treatment (Qiagen), according to the manufacturer's guideline. RNA concentration was determined using the Qubit 2.0 (Life Technologies). RNA quality was measured using the Agilent Bioanalyzer and produced RIN values of >8.0.

Transfection with LNA antisense

On the day of transfection, 5 µL of HiPerFect (Qiagen) was mixed with 200 µL of media without antibiotics, serum or antifungals to prepare the transfection mix. LNA GapmeRs were added to 200 µL of the transfection mix at a final concentration of 30 nM, placed in 12-well plates and incubated for minimum 10 mins at room temperature. Fibroblasts were then seeded at a density of $5x10^5$ cells per well in 200 µL of growth media and incubated with the transfection mixes at 37^oC, 5% (v/v) CO_2 overnight. The next day, 800 µL of media (0.1% FBS) was added to the wells to dilute the lipid-LNA complexes and reduce the toxicity of the reaction. The cells were stimulated with 3 ng/ml of IL-1β and incubated for 24 hrs before harvesting for RNA extraction and ELISA (Enzyme-linked immunosorbent assay) analysis. LNA GapmeR sequences: Negative Control LNA1 - TCATACTATATGACAG; Negative Control LNA2 - GACGGTAAGTAGGCGA; IL7AS LNA1 – GGCGTGAGAGTAAAGC; IL7AS LNA2 – GTGCTTAGGCTTAGAG; MIR3142HG LNA1 **–** GTAAACGAGTAGCAGC; MIR3142HG LNA2 – GAACATGGTTACGTGT.

Measurement of IL-6, IL-8 and CCL2 release

Supernatants of cultured lung fibroblasts were collected and used to assess secretion of IL-6 and CCL2, using the DuoSet ELISA (DY206 and DY279) Development System Kits (R&D Systems Europe, UK) and IL-8 (Ready-SET-Go!®, eBioscience), following the manufacturer's instructions.

Quantitative PCR validation of lncRNA expression

For quantitative PCR (qPCR), cDNA libraries were prepared from total RNA using the High capacity cDNA RT kit (Applied Biosystems, Life Technologies, 4368813). Expression of mRNAs and lncRNAs were determined by qPCR using the SYBR® Green PCR mix (Applied Biosystems; primers were obtained from Sigma-Aldrich). For analysis, the 2−(ΔΔCt) method was used to determine relative-quantities of individual mRNAs and lncRNAs which were normalized to 18S ribosomal RNA. qRT-PCR primer sequences: 18S – AAACGGCTACCACATCCAAG (Forward), CCTCCAATGGATCCTCGTTA (Reverse); IL7AS – GTGGACGATGCCAAGTCGT (Forward), AGGTGCATGTACAGCAGACG (Reverse); MIR3142HG – AGCTTGGAAGACTGGAGACAG (Forward), TCACAGGAACTCACACTCCT (Reverse).

Transcriptome analysis of IL-1β-stimulated lung fibroblasts

Total RNA was extracted from lung fibroblast exposed to either buffer (controls) or 3 ng/ml of IL-1β for 6 hrs. Paired-end 75bp sequencing data were obtained using the Illumina HiSeq4000 at the Oxford Genomics Centre at the Wellcome Centre for Human Genetics (funded by Wellcome Trust grant reference 203141/Z/16/Z). The paired end reads were aligned to the human reference genome (hg38) using Hisat2 (version 2.0.4) (26,27) using the following command line options: hisat2 -q --dta --rna-strandness FR –x <reference-genone.gtf> -1 <forward_strand.fa> -2 <reverse-strand file.fa> –S <output.sam>. Output SAM files were then sorted and converted to BAM files (samtools sort -@ 8 –o output.bam output.sam) and indexed (samtools index –b output.bam) in Samtools (28). The profile of gene expression (using the Gencode v27 database and additional novel lncRNA (25)) in the BAM files for each samples were determined using Stringtie (29): stringtie <sample.BAM> -G <GenCodev26.gtf> -o <samples.gtf> -e -A <sample.txt>. The differential expression of gene derived from Gencode v27 and our recently generated list of novel lncRNA implicated in the innate immune (25) was assessed with the geometric option (DESeq) in Cuffdiff v2.2.1.3 (part of the Cufflinks suite (30)) using a significance threshold of q <0.05. The command line options were as follows: cuffdiff --FDR=0.05 --min-alignment-count=10 --library-norm-

method=geometric --dispersion-method=pooled -u <reference_genome.gtf> <control_1.bam>,<control_x.bam> <activated_1.bam>,<activated_x.bam> -o <output_file_name>.

Principle Component Analysis and Hierarchical Clustering

The abundance of Gencode v27 defined genes in individual samples was defined as the fragments per kilobase exon per million reads mapped (FPKM) and determined using Stringtie (RNA) (see above). PCA and hierarchical clustering on Gencode v27 protein coding genes demonstrating an expression >1 FPKM was performed using Genesis (v1.7.7) (31). Data were log2 transformed following the addition of 1 FPKM. The threshold for reporting gene expression at FPKM >1 is based upon the ability to validate sequencing data using qRT-PCR**.**

Data Access

RNA sequencing data for control and IL-1β-stimulated fibroblasts can be obtained at the Gene Expression Omnibus at GSEXXXXX.

Supplementary data (provided on USB memory stick).

3.4. Results

Differential expression of protein-coding genes in IL-1β-stimulated lung fibroblasts

Initial studies were undertaken to examine the IL-1β-induced changes in mRNA expression in control fibroblasts (Supplemental Table 1). Selecting only those mRNAs showing a fold-change >2, absolute change of 1 FPKM and $q < 0.05$, we identified 453 up-regulated and 261 down-regulated mRNAs (Figure 1A). As might be expected, pathway analysis (DAVID Bioinformatics resources 6.8; https://david.ncifcrf.gov/home.jsp) showed that the up-regulated mRNAs were associated with multiple inflammatory pathways (Figure 1B). No pathways were highlighted with the down-regulated mRNAs.

Profile of primary miRNA and lncRNAs expression in lung fibroblasts

Before investigating those lncRNAs that were differentially expressed in response to IL-1β, we initially examined the profile of lncRNA expression in non-stimulated control fibroblasts. As a result of the difficulty in assigning sequencing data to either the original mRNA or pseudogenes during alignment, the pseudogenes were excluded from this analysis. Using a cut-off of FPKM > 1 to identify those expressed at physiologically relevant levels, we identified 484 lncRNAs that could be divided into 225 lincRNAs and 259 antisense (Supplemental Table 2), $q < 0.05$. Amongst the most highly expressed lncRNAs were NORAD (Non-coding RNA activated by DNA damage), a lncRNA that's binds Pumilo proteins and regulates genomic stability (32,33), MIR4435-2HG, a host gene (primary miRNA) for miR-4435, two small nucleolar host genes (SNHG7/8) and FENDRR (FOXF1 adjacent non-coding developmental regulatory RNA) a lncRNA involved in heart and body wall development (34) (Figure 1C).

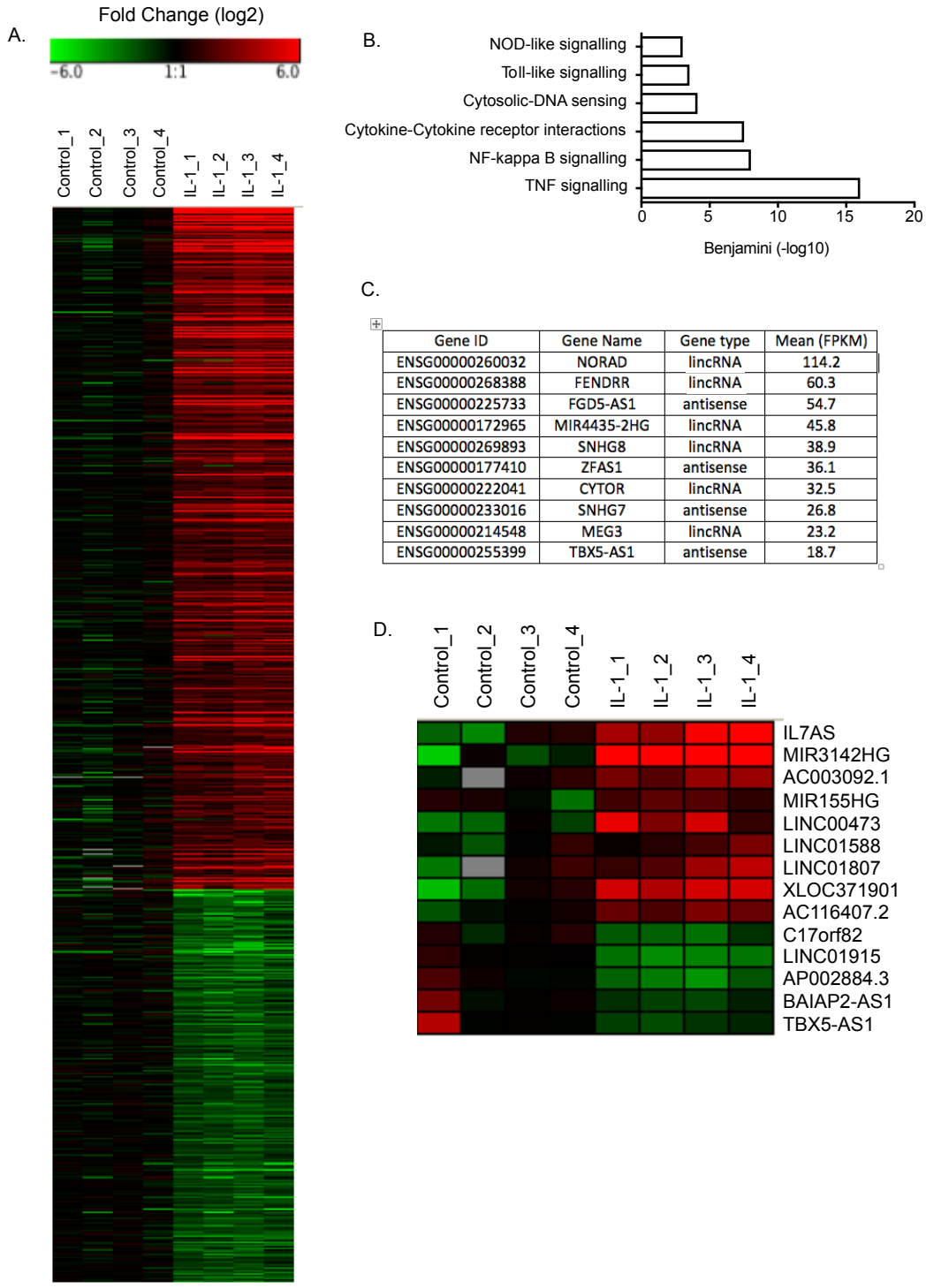

Figure 1. Differential expression of mRNAs and lncRNAs following IL-1β-stimulation of control lung fibroblasts. (A) Heat map showing the differential expression of mRNAs in control fibroblasts following IL-1β stimulation for 6 hrs. (B) Pathway analysis of up-regulated mRNAs. (C) Top 10 most highly expressed lncRNA in non-stimulated control fibroblasts. (D) Heat map showing the differential expression of lncRNAs in control fibroblasts following IL-1β stimulation for 6 hrs.

Differential expression of long noncoding RNAs in IL-1β-stimulated lung fibroblasts

To identify those lncRNAs that might mediate the inflammatory response, we compared the profile of lncRNA expression in control and IL-1β-stimulated fibroblasts at 6 hrs. Using the same criteria as was applied to mRNAs ($q < 0.05$, fold change > 2 and absolute expression change > 1 FPKM), we showed differential expression of 12 lincRNAs and 2 antisense, of which 7 were up-regulated and 5 down-regulated (Figure 1D). Of these, IL7AS and MIR3142HG showed the largest fold-changes (IL7AS (48-fold) and MIR3142HG (157-fold)) and absolute-change (IL7AS (9.2 FPKM) and MIR3142HG (5.5 FPKM)) (Supplemental Table 3). These increases in the expression of IL7AS and MIR3142HG were confirmed by examination of the sequencing data in the IGV genome browser (Figure 2) and qRT-PCR analysis (Figure 3A/B). Of relevance, although MIR3142HG is the host gene for miR-3142, this also contains miR-146a, a widely reported regulator of inflammation and the immune response (11,12). Examination of the sequencing coverage in the IGV genome browser indicates that processing of this MIR3142HG will produce miR-146a and not miR-3142 (Figure 2). Also amongst the differentially expressed lncRNAs is MIR155HG, the host gene (primary miRNA transcript) for miR-155, another regulator of the inflammation (11,12).

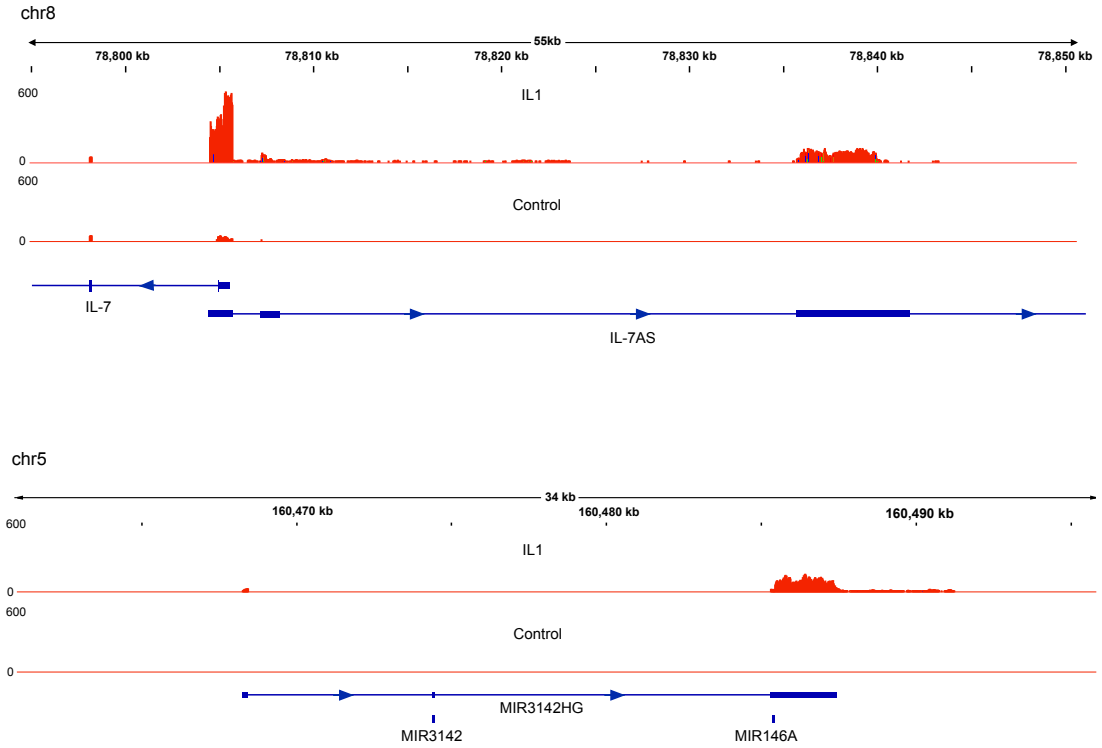

Figure 2. IL-1β-induced expression of IL7AS and MIR3142HG in control fibroblasts. Aligned sequencing data (merged BAM files) showing IL7AS and MIR3142HG from control and IL-1β-stimulated control fibroblasts was visualised using the IGV genome browser (https://software.broadinstitute.org/software/igv/).

IL7AS and MIR3142HG regulate the IL-1β-induced inflammatory response

In subsequent studies we investigated the function of IL7AS and MIR3142HG in lung fibroblasts during the IL-1β-induced inflammatory response. To this end, we identified 2 locked nucleic acid based (LNA) antisense sequences against IL7AS (Figure 3A) and MIR3142HG (Figure 3B) that produced 50-85% knockdown following overnight transfection into fibroblasts and stimulation with IL-1β for 24 hrs. Following exposure to IL-1β, we observed increased release of the inflammatory mediators IL-6 (Figure 3C/D), IL-8 (Figure 3E/F) and CCL2 (Figure 3 G/H), Knockdown of IL7AS enhanced the

release of IL-6 (Figure 3C) but had no effect upon IL-8 or CCL2 (Figure 3E/G). In contrast, MIR3142HG had no effect upon IL-6 (Figure 3D) but significantly reduced the release of IL-8 and CCL2 (Figure 3F/H). This indicates that IL7AS and MIR3142HG differentially regulate the release of inflammatory mediators during IL-1β-induced activation, with IL7AS being a negative regulator of IL6 release and MIR3142HG a positive regulator of IL8 and CCL2 release.

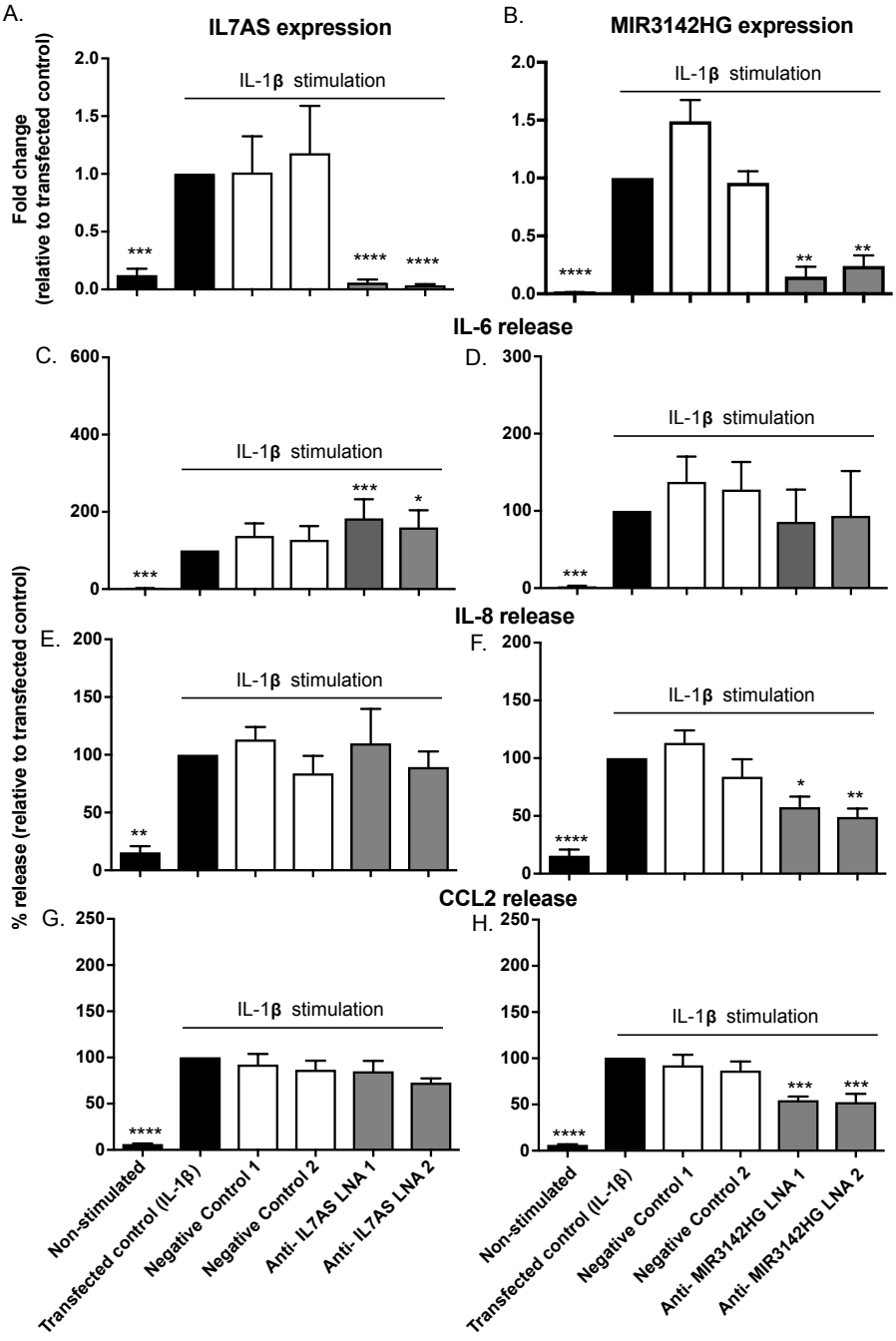

Figure 3. IL7AS and MIR3142HG regulates the IL-1β-stimulated inflammatory response in control fibroblasts. Control fibroblasts were

transfected overnight with LNA antisense sequences against IL7AS (A/C/E/G) and MIR3142HG (B/D/F/H) or scrambled (negative) controls. Cell were then stimulated with IL-1β for 24 hrs prior to isolation of RNA and measurement of IL7AS (A) or MIR3142HG (B) by qRT-PCR or measurement of supernatant IL-6 (C/D), IL-8 (E/F) and CCL2 (G/H) by ELISA. Data represents the mean +/- SEM of five control individuals. Statistical significance was performed using the repeat measures 1-way analysis of variance (ANOVA) with a Dunnett's test where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001.

IL7AS and MIR3142HG and the IL-1β-induced inflammatory response in IPF fibroblasts

Previous studies have demonstrated differences in the phenotypic responses between lung fibroblasts derived from control and IPF patients including a recent meta-analysis of microarray data showing repression of inflammation and immune pathways in IPF (35). Initial comparison of raw CT values obtained from qRT-PCR showed no difference in the baseline expression of IL7AS (Control = 23.3 +/- 1.0; IPF = 22.2 +/- 1.1) and MIR3142HG (Control = 26.35 +/- 0.7; IPF = 27.8 +/- 0.5) between control and IPF fibroblasts. Interestingly, although there was no significant difference in the IL7AS expression between control and IPF cells following IL-1β stimulation (Control = 18.6 +/- 0.8; IPF = 19.4 +/- 1.3), there was an approximate 8-fold reduction in the MIR3142HG production in IPF (Control = 19.3 +/- 0.6; IPF = 22.6 +/- 1.0 : p=0.0225).

Using LNA-based antisense, we once again demonstrated 50-85% knockdown of IL7AS (Figure 4A) and MIR3142HG (Figure 4B) following overnight transfection into IPF fibroblasts and stimulation with IL-1β for 24 hrs. As with control cells, knockdown of IL7AS caused a significant increase in IL-6 release (Figure 4C) but had no effect upon IL-8 (Figure 4E) or CCL2 production (Figure 4G). Interestingly, MIR3142HG knockdown had no effect upon release of either IL-6, IL-8 or CCL2, which might reflect the 8-fold reduction in the IL-1β-induced increased in MIR3142HG seen in IPF fibroblasts (Figure 4D/F/H).

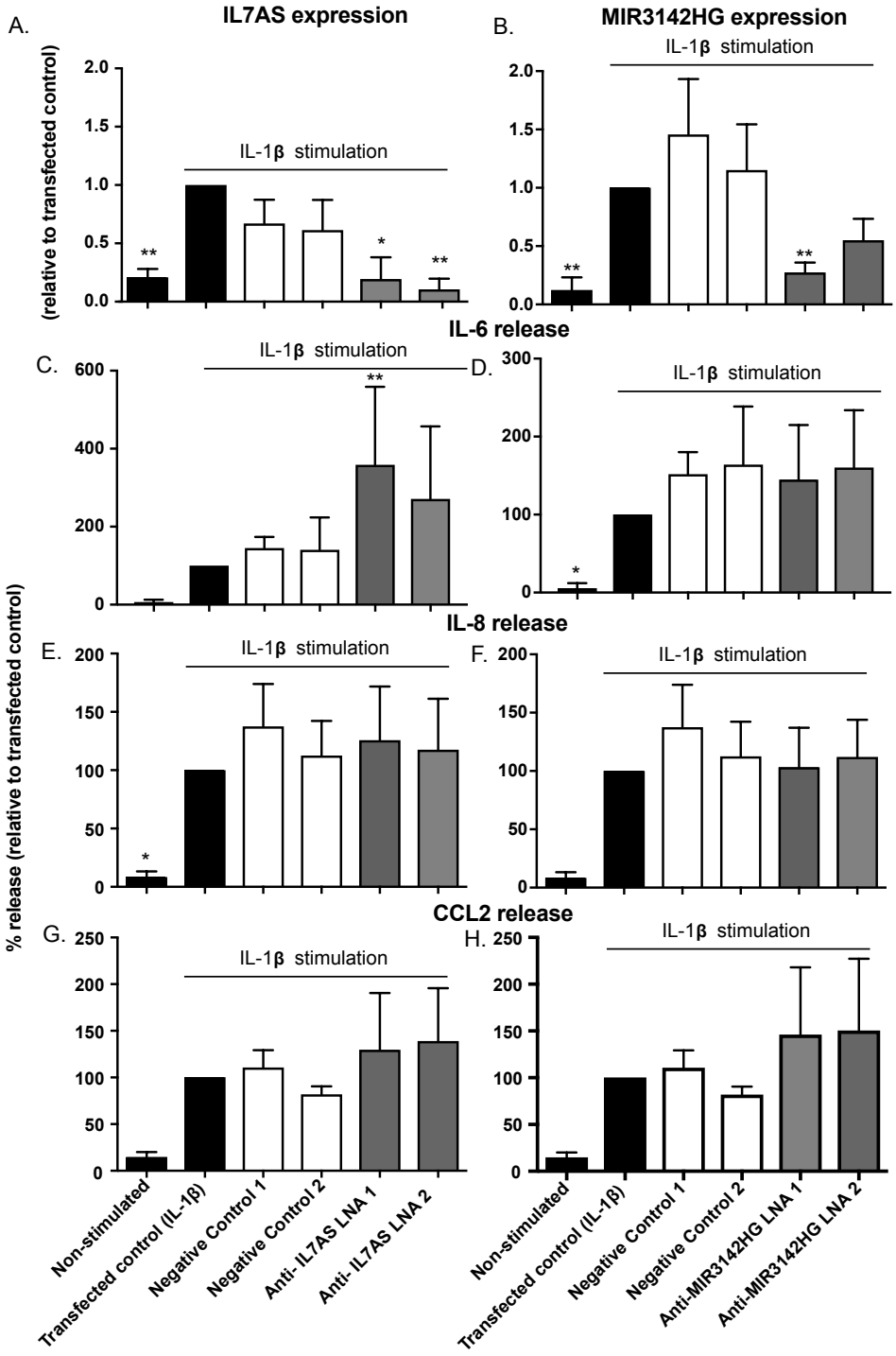

Figure 4. IL7AS but not MIR3142HG regulates the IL-β-stimulated inflammatory response in IPF fibroblasts. IPF fibroblasts were transfected overnight with LNA antisense sequences against IL7AS (A/C/E/G) and MIR3142HG (B/D/F/H) or scrambled (negative) controls. Cell were then stimulated with IL-1β for 24 hrs prior to isolation of RNA and measurement of IL7AS (A) or MIR3142HG (B) by qRT-PCR or measurement of supernatant IL-6 (C/D), IL-8 (E/F) and CCL2 (G/H) by ELISA. Data represents the mean +/- SEM of five IPF individuals. Statistical significance was performed using

the repeat measures 1-way analysis of variance (ANOVA) with a Dunnett's test where * = p <0.05, ** = p <0.01, *** = p <0.001 and **** = p <0.0001

3.5. Discussion

We have for the first time examined the changes in lncRNAs expression following activation of human lung fibroblasts with the potent pro-inflammatory mediator, IL-1β. This demonstrated differential expression of 14 lncRNAs including IL7AS, a syntenically conserved antisense that overlaps and is expressed in a bi-directional manner with the promoter of IL-7 (25), as well as the host genes for two miRNAs, MIR3142HG (miR-146a) and MIR155HG (miR-155). In subsequent functional studies, we employed LNA antisense knockdown to examine the role of IL7AS and MIR3142HG, the two most highly induced lncRNAs, during the IL-1β-induced inflammatory response. As we have previously reported (25,36), IL7AS knockdown was shown to increase IL-6 release from both control and IPF-derived fibroblasts, indicating that this is a negative regulator. However, in contrast to these earlier reports, this action appear to be selective for IL-6, as there was no effect upon the release of IL-8 and CCL2 (25,36). Given that MIR3142HG is the host genes for miR-146a, it might be speculated that knockdown would lead to a reduction in both the full length lncRNAs transcript and processed miR-146a, a well-characterised miRNA that is thought to negatively regulated the inflammatory response through down-regulation of TRAF6 and IRAK1 (37,38). Unexpectedly, MIR3142HG knockdown in control fibroblasts resulted in a reduction in IL-1β-induced IL-8 and CCL2 release, with no effect upon IL-6, indicating that MIR3142HG/miRNA-146a is a positive regulator of the inflammatory response in human lung fibroblasts. Of relevance, we have also observed cell-type specific actions with IL7AS, this lncRNA showing negative regulation of the inflammatory response in monocytes and chondrocytes but positive regulation in airway epithelium (25,36). Unfortunately, it is not possible to separate the actions of MIR3142HG from those of miRNA-146a since miRNA knockdown would also be expected to reduce the levels of the host gene. Finally, MIR3142/miR-146a knockdown was shown to have no effect upon the IL-1β-induced release of IL-6, IL-8 and CCL2 from IPF-derived

lung fibroblasts. However, this is likely to have resulted from 8-fold reduction in the IL-1β-induced expression of MIR3142/miR-146a seen in IPF fibroblasts. The underlying cause for this reduction in MIR3142/miR-146a expression in IPF fibroblasts in unknown although this indicates that changes in lncRNA/miRNA expression occurs in disease and that these might under be responsible for pathology.

In conclusion, this report has catalogued those lncRNAs that are differentially expressed following IL-1β-stimulated activation of human lung fibroblasts and demonstrated that 2 lncRNAs, IL7AS and MIR3142HG regulate the inflammatory response. Subsequent studies will be required to examine the role of the additional lncRNAs and to elucidate the mechanism of action of those lncRNAs that are functional relevant.

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4. Chapter 4 – Effects of pirfenidone and nintedanib upon the fibrotic, proliferative and inflammatory response in human lung fibroblasts

Statement of Authorship Form

This declaration concerns the article entitled:

Effects of pirfenidone and nintedanib upon the fibrotic, proliferative and inflammatory response in human lung fibroblasts									
Publication status (tick one)									
draft manuscript		**Submitted**		**In review**	√	**Accepted**		**Published**	
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Candidate's contribution to the paper (detailed, and also given as a percentage).	The candidate contributed to/ considerably contributed to/predominantly executed the… Formulation of ideas (50%): Marina Hadjicharalambous and Mark A. Lindsay decided equally on direction of the project based on previous literature and research. Design of methodology (70%): Experiments were mainly designed by Marina Hadjicharalambous. Mark A. Lindsay, Benoit T. Roux and Deborah L. Clarke contributed equally. Experimental work (100%): All experimental work was undertaken by Marina Hadjicharalambous. Presentation of data in journal format (95%): Marina Hadjicharalambous was the main author of the paper with corrections and suggestions offered by the co-authors.								
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.								
Signed						**Date**			

Effects of pirfenidone and nintedanib upon the fibrotic, proliferative and inflammatory response in human lung fibroblasts

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Key Words: fibroblasts; proliferation; fibrosis; nintedanib; pirfenidone

4.1. Abstract

Aim: Idiopathic pulmonary fibrosis (IPF) is a fatal progressive chronic disease characterised by scar tissue accumulation in the lungs leading to impaired gas exchange and restricted ventilation. The aetiology and pathogenesis of the disease are still unknown, although lung fibroblast have been strongly associated with the development and progression of the disease. Pirfenidone and nintedanib have recently been licensed for the treatment of IPF although their mechanism of action is currently unclear. In this report, we have examined the role pirfenidone and nintedanib in the phenotypic response of lung fibroblasts obtained from control and IPF patients.

Materials and Methods: Control and IPF fibroblasts were cultured *in vitro* and the fibrotic, proliferative and inflammatory response was determined in the presence or absence of pirfenidone or nintedanib by measuring TGF-β1-induced PAI-1 release, PDGF-AB-induced changes in cells number using the CCK-8 viability assay and IL-1β-induced IL6 release, respectively.

Results: Pirfenidone had no significant effect upon the response to TGF-β1, PDGF-AB and IL-1β in neither control or IPF fibroblasts. Nintedanib inhibited TGF-β1-induced PAI-1 release and PDGF-AB induced proliferation but had no effect upon IL-1β-induced IL-6 release. Inhibition of fibrosis and proliferation by nintedanib was seen at lower concentrations in IPF compared to control fibroblasts.

Conclusions: Nintedanib, but not pirfenidone, attenuated the TGF-β1-induced fibrotic and PDGF-AB proliferative response in human lung fibroblasts and was more effective in IPF compared to control patients. Neither drugs effected the IL-1β-induced inflammatory response.

4.2. Introduction

The secretion of cytokines and growth factors during the wound healing response is thought to influence the activity of fibroblasts and has been associated with the development of idiopathic pulmonary fibrosis (IPF), a progressive chronic lung disease of unknown aetiology (1). It is now become apparent that both the epithelial and inflammatory pathways play a role in the development and progression of IPF (2,3). TGF-β1 (transforming growth factor β-1), PDGF (platelet-derived growth factor) and IL-1β are amongst the cellular mediators that have been shown to regulate the activity of fibroblasts during the tissue repair response as they have previously been implicated in the induction of fibrogenesis, proliferation and inflammation in IPF (4-6).

TGF-β1 is the most well studied pleiotropic pro-fibrotic cytokine and is thought to be the key player in the pathogenesis of pulmonary fibrosis (7). TGF-β1 plays a central role in events driving the fibrotic response such as fibroblast activation and myofibroblast differentiation (8,9), extracellular matrix (ECM) production (10,11) and epithelial-mesenchymal-transition (EMT) (12). Elevated expression of PDGF, a potent fibroblast mitogen and chemoattractant, is thought to be another 'master switch' in the development of IPF (13). PDGF isoforms were shown to drive the proliferation (14) and migration (15) of fibroblasts in fibrosis as well as the secretion of inflammatory cytokines (16). The role of IL-1β in the development of IPF remains unclear as not much is known about the activity of this pro-inflammatory cytokine during fibrosis. Kolb *et al*. has reported an indirect pro-fibrotic activity for IL-1β *in vivo* (17), while increased levels were detected in the bronchoalveolar lavage (BAL) of IPF patients (18). Interestingly, although it was shown to directly stimulate collagen expression in fibroblasts (19), another report has shown inhibition (20). A more recent study suggests that IL-1β has an anti-fibrotic effect on dermal and lung fibroblasts *in vitro* (21).

The lack of understanding regarding the underlying mechanisms of IPF makes the development of effective drug treatments particularly challenging. Nintedanib (BIBF1120) was approved for use in 2014 after the completion of

the INPULSIS trials (22). This small molecule was shown to slow the progression of IPF through inhibition of multiple receptor tyrosine kinases including vascular endothelial growth factor (VEGFR), fibroblast growth factor (FGFR) and platelet-derived growth factor (PDGFR) both *in vivo* (23,24) and *in vitro* (25,26). Following the satisfactory results of the ASCEND trial (27), pirfenidone was also approved in 2014 as the first specific anti-fibrotic therapy. Pirfenidone is a novel anti-fibrotic, anti-oxidant and anti-inflammatory drug which is thought to reduce TGF-β1 expression (28-30), although its exact mechanism of action is still unclear. Pirfenidone was shown to attenuate fibrosis by reducing proliferation as well as α-smooth muscle actin (α-SMA) and collagen levels in fibroblasts *in vitro* (26,31,32). Pirfenidone has also demonstrated anti-fibrotic activities in the bleomycin animal models, through attenuating fibrocyte recruitment to the lungs and the secretion of several cytokines (33,34), as well as PDGF (35) and TGF-β1 (28) synthesis.

The secretion of these fibrotic mediators during tissue repair has been established as a crucial element in the development of IPF, yet more information is required on how each of these compounds individually influence lung fibroblast activation. We have therefore examined the effect of the nintedanib and pirfenidone upon the fibrotic, proliferative and inflammatory responses in lung fibroblasts obtained from both control and IPF patients.

4.3. Materials and Methods

Fibroblast source and cell culture

Control (age = 47 ± 7y; 1 male and 2 females) and IPF fibroblasts (age = 63 ± 1y; 2 males and 1 female) were obtained from Professor Carol Ferghali-Bostwick (Medical University of South Carolina, USA) and the Coriell Institute of Medical Research (Camden, New Jersey, USA). Neither the control or IPF patients had a history of smoking. Isolation of lung fibroblasts was initiated using explants of minced lung tissue. Fibroblasts were cultured in DMEM (high glucose, pyruvate) growth media (11995-073, ThermoFisher) supplemented with 10% (v/v) FBS (Fetal Bovine Serum) (11550356, ThermoFisher), 1% (v/v)

Penicillin-Streptomycin (11548876, ThermoFisher) and 0.1% (v/v) Fungizone (15290-018, ThermoFisher). All cultures were maintained in a 37^{o}C, 5% (v/v) CO_2 humidified incubator. Upon reaching approximately 80-90% confluency cells were washed in sterile 1x PBS (Phosphate Buffered Saline) (P5493, Sigma-Aldrich) followed by treatment with StemPro® Accutase® cell detachment solution (11599686, ThermoFisher). All experiments were performed using cells plated at passage 6 to 7.

Plating and treatments of fibroblasts for pharmacological studies

1 x 10^4 cells were plated in 96-cell culture wells on day 1 and allowed to rest overnight. On day 2, cells were serum-deprived by reducing FBS to 0.1% in 200 µl of media before treatment application. Fibroblasts were then treated with the required concentration of TGF-β1 (recombinant human, expressed in Chinese hamster ovary cell line, R&D systems, 240-B-002/CF), IL-1β (recombinant, expressed in *E. coli*, Sigma-Aldrich, I9401-5UG), pirfenidone ($C_{12}H_{11}NO$, Sigma-Aldrich, P2116) and nintedanib (BIBF1120, $C_{31}H_{33}N_5O_4$, Cayman Chemical, 11022) and incubated for the required time before supernatants were collected for protein quantification assessment.

Measurement of IL-6 and PAI-1 release

Supernatants of cultured lung fibroblasts were collected and used to assess secretion of IL-6 and PAI-1, using the DuoSet ELISA (Enzyme-linked immunosorbent assay, DY206 and DY1786) Development System Kits (R&D Systems Europe, UK) following the manufacturer's instructions. Absorbance was measured at 450 nm with wavelength correction at 570 nm using a microplate reader (Fluostar Optima, BMG Labtech).

Cell Proliferation

Lung fibroblast proliferation was evaluated by measuring cell viability with the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich). Cells (5000) were seeded in a 96-well plate and incubated overnight in 100 µl growth media (10% FBS). The following day, the cells were serum-deprived and treated with the indicated concentration of PDGF-AB (recombinant human, *E. coli* derived, R&D systems, 222-AB-010), pirfenidone ($C_{12}H_{11}NO$, Sigma-Aldrich, P2116) and

nintedanib (BIBF1120, $C_{31}H_{33}N_5O_4$, Cayman Chemical, 11022) and incubated for the indicated time. Before the end of each assay, 10 µl of the CCK-8 solution was added into a final volume of 100 µl growth media and incubated for 2 hours before absorbance was measured using a microplate reader (Fluostar Optima, BMG Labtech). The absorbance wavelength was measured at 450 nm and 600 nm which was then subtracted during data analysis.

Statistical Analysis

All statistical analysis and graphs were generated using GraphPad Prism 7 software. Statistical significance was determined using repeated measures ANOVA with a Dunnett's multiple comparisons test (each column is compared to the positive control column), where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Negative control columns were not included in the statistical comparison tests.

4.4. Results

To determine the mechanism of action of pirfenidone and nintedanib in fibrosis, proliferation and inflammation, we assessed their concentration dependent action upon TGF-β1-induced PAI-1 release, PDGF-AB-induced cell number and IL-1β-stimulated IL-6 release at 72 hours (hrs), respectively. In addition, we compared their action in lung fibroblasts derived from control and IPF patients to assess whether they had differential action. The concentration ranges of pirfenidone (10 uM – 1000 uM) and nintedanib (3 nM – 3000 nM) were selected based on previous studies investigating their biological actions (25,26,36-38).

Effect of pirfenidone and nintedanib on the fibrotic response

PAI-1 levels have been shown to be increased in the lungs of patients with diverse lung diseases including IPF, where it is considered to exert a pro-fibrogenic effect (39). Furthermore, TGF-β1 was previously shown to increase PAI-1 expression in several cell types including lung fibroblasts and the release of PAI-1 is considered to be a marker of fibrosis (40-43).

Exposure to TGF-β1 induced a significant increase in release of PAI-1 from both control and IPF fibroblasts. Pirfenidone treatment had no effect upon non-stimulated or TGF-β1-induced PAI-1 release from either control or IPF fibroblasts (Figure 1A). Although nintedanib had no impact upon PAI-1 release from non-stimulated cells, it attenuated TGF-β1-induced PAI-1 generation from both control (46.6%) and IPF (56.7%) fibroblast at 3000nM (Figure 1B). It appeared to be more effective against IPF fibroblast since significant inhibition was also seen at 300nM (75%) and 1μM (68%). These observations indicate that nintedanib but not pirfenidone, inhibits the fibrotic response and that IPF fibroblasts are more sensitive to the action of the drug.

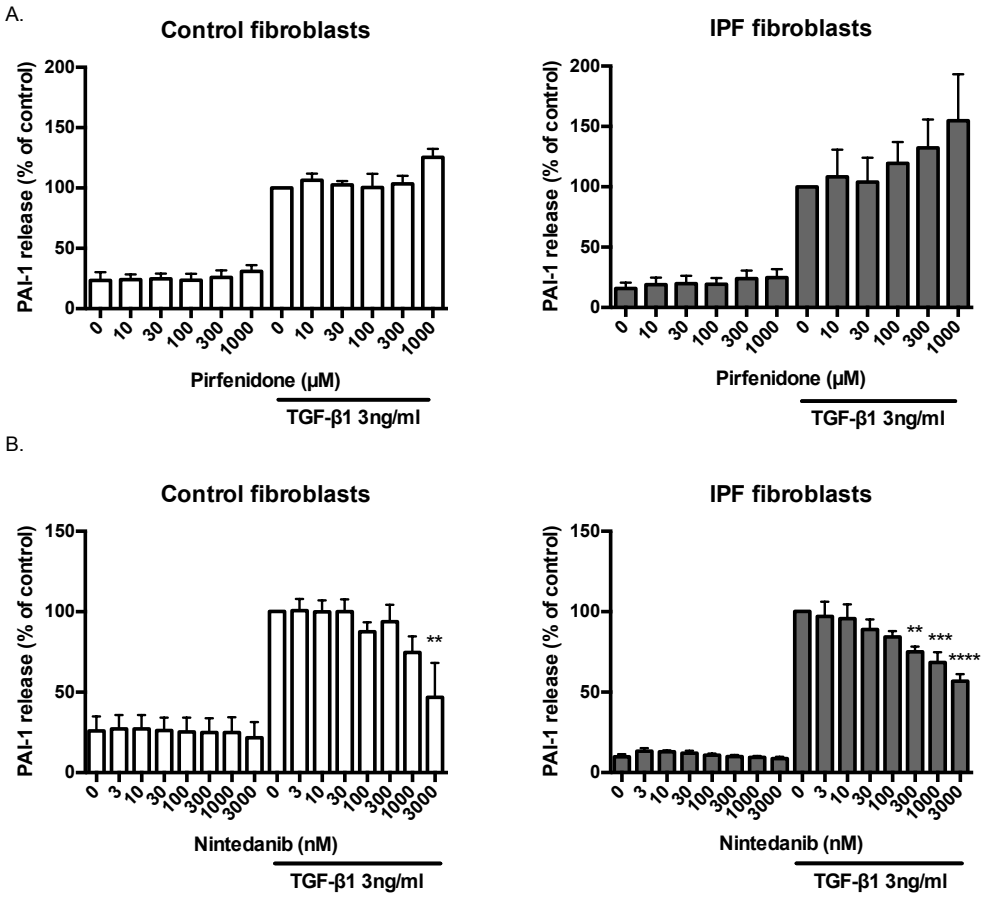

Figure 1. Concentration dependent effect of pirfenidone and nintedanib upon PAI-1 release from control and IPF lung fibroblasts. Human control and IPF lung fibroblasts were exposed to the indicated concentrations of pirfenidone **(A)** and nintedanib **(B)** for 72 hrs with and without 3 ng/ml TGF-β1. Each column represents the mean ± SEM of 3 independent patients of which 3 replicates were used to calculate the mean of each data point. All columns are compared to the positive control treated with TGF-β1 and no antagonist. Columns treated with pirfenidone or nintedanib alone serve as negative controls.

Effect of pirfenidone and nintedanib on proliferative response

PDGF-A and PDGF-B are the predominantly expressed isoforms of the PDGF family implicated in fibrosis where they are thought to promote fibroblast proliferation (24,44)**;** while evidence support the concept that cross-talk between TGF-β1 and PDGF may further modulate the progression of irreversible scarring (45).

As expected, incubation of lung fibroblasts in the presence of PDGF-AB promoted cell proliferation in both control and IPF fibroblasts (Figure 2). Pirfenidone had no significant effect upon non-stimulated or PDGF-AB-induced cell proliferation from either control or IPF fibroblasts (Figure 2A). Nintedanib had also no impact upon cell proliferation from non-stimulated cells but attenuated PDGF-AB-induced proliferation from both control and IPF fibroblast in a concentration dependent manner, reducing cell counts to 16% and 32% at 3 μM, respectively (Figure 2B). Notably, nintedanib significantly inhibited proliferation at lower concentrations in IPF fibroblasts starting at 30 nM compared to 300 nM in control fibroblasts. These observations indicate that nintedanib but not pirfenidone, attenuates proliferation and that IPF fibroblasts are more sensitive to the action of the drug.

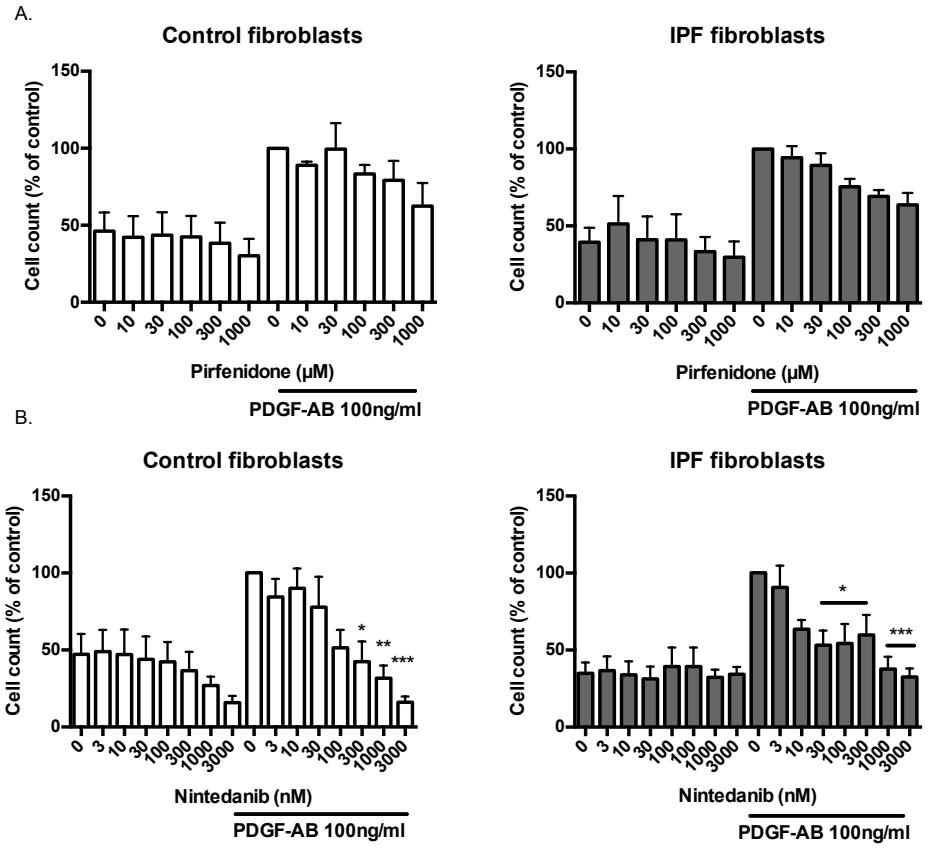

Figure 2. Concentration dependent effect of pirfenidone and nintedanib on cell proliferation in control and IPF lung fibroblasts. Human control and IPF lung fibroblasts were exposed to the indicated concentrations of pirfenidone **(A)** and nintedanib **(B)** for 72 hrs with and without 100 ng/ml PDGF-AB. Each column represents the mean ± SEM of 3 independent patients of which 3 replicates were used to calculate the mean of each data point. All columns are compared to the positive control treated with PDGF-AB and no antagonist. Columns treated with pirfenidone or nintedanib alone serve as negative controls.

Effect of pirfenidone and nintedanib on inflammatory response

IL-1β was previously shown to drive IL-6 expression in orbital and synovial fibroblasts *in vitro* (46,47), however not much is known regarding its effect on lung fibroblasts. As anticipated, exposure to IL-1β induced high levels of IL-6 cytokine release in both control and IPF lung fibroblasts (Figure 3). Neither pirfenidone or nintedanib had an effect upon non-stimulated or IL-1β-induced IL-6 release from either control or IPF fibroblasts (Figure 3) and indicates that neither drug acts by attenuating the inflammatory response.

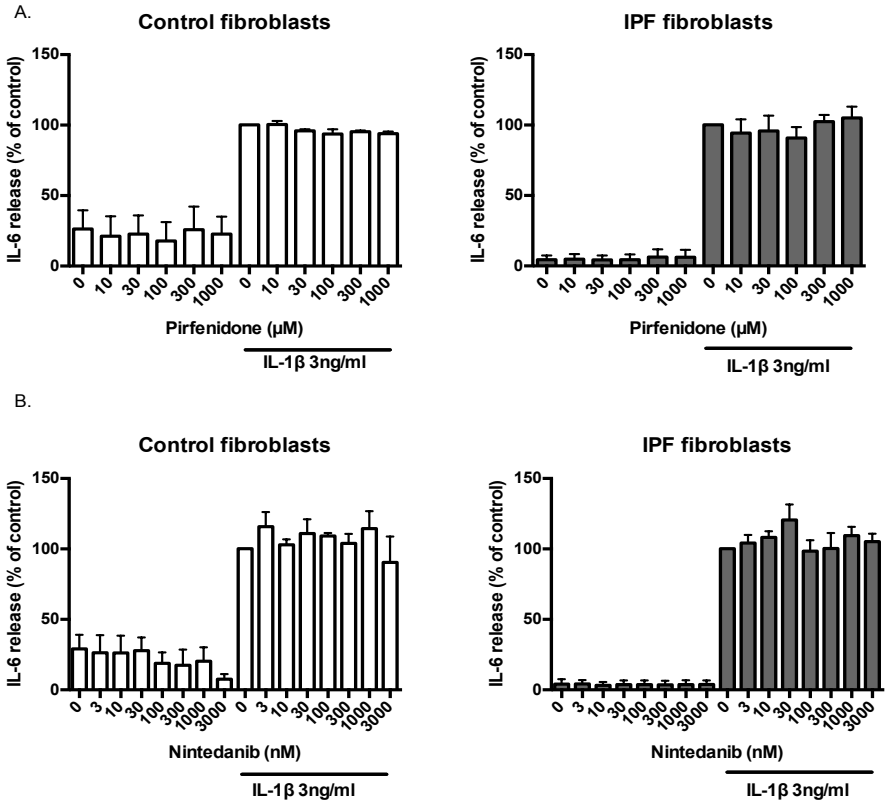

Figure 3. Concentration dependent effect of pirfenidone and nintedanib upon IL-6 release from control and IPF lung fibroblasts. Human control and IPF lung fibroblasts were exposed to the indicated concentrations of **(A)** pirfenidone and **(B)** nintedanib for 72 hrs with and without 3 ng/ml IL-1β. Each column represents the mean ± SEM of 3 independent patients of which 3 replicates were used to calculate the mean of each data point. All columns are compared to the positive control treated with IL-1β and no antagonist. Columns treated with pirfenidone or nintedanib alone served as negative controls.

4.5. Discussion

In this report, we examined the concentration-dependent effects of nintedanib and pirfinedone in TGF-β1, PDGF-AB and IL-1β activated lung fibroblasts by measuring PAI-1, cell viability and IL-6, respectively. The highest concentration of nintedanib was 3000 nM, which was higher than the 1000 nM employed in previous studies *in vitro* (24-26). We also initially employed a supra-maximal concentration pirfenidone at 10000 μM but as a result of toxicity, reduced this to 1000 μM suggested by other authors (26,31).

The TGF-β1-induced PAI-1 release of lung fibroblasts was not inhibited by pirfenidone. The inability of pirfenidone to reduce the TGF-β1-induced response contrasts observations by other groups showing pirfenidone inhibiting the expression of TGF-β1-induced proteins such as collagen, α-smooth muscle actin (α-SMA) and connective tissue growth factor (29,31,48). In contrast, nintedanib significantly reduced PAI-1 release of lung fibroblasts, with a more potent effect on IPF lung fibroblasts. This finding is in line with previous studies demonstrating the inhibitory activity of nintedanib on TGF-β1-induced fibronectin and α-SMA production (26,49).

Treatment with pirfenidone showed no significant reduction in PDGF-AB-induced cell proliferation. Significant inhibition in cell viability of control fibroblasts was noted upon treatment with higher concentrations of pirfenidone although this effect is likely to be mediated by the cellular toxicity. Although pirfenidone is not primarily known as an anti-proliferative agent, it was found to reduce lung fibroblast proliferation at higher concentrations *in vitro* by Conte and co-workers (31) as well as cardiac fibroblasts (50), stromal cells (26) and tenon's fibroblasts (51). Nintedanib as a potent inhibitor of VEGFR, FGFR and PDGFR, effectively reduced cell viability in both control and IPF fibroblasts. Notably, the action of nintedanib was observed at lower concentrations in IPF lung fibroblasts compared to healthy controls. Interestingly, a previous study by Hostettler *et al.* suggests greater sensitivity of nintedanib by control fibroblasts (25) whereas a more recent study by

Lehtonen *et al*. does not report a significant difference between stromal cells derived from control and IPF lungs (26).

Neither pirfenidone nor nintedanib inhibited the IL-1β-induced release of IL-6 from control and IPF lung fibroblasts. Although pirfenidone demonstrated an inhibitory activity towards IL-6 levels in previous studies (52-54), no other evidence regarding the inhibition of the IL-1β-induced release of this pro-inflammatory cytokine exist by either of these antagonists.

In summary, the data presented in this study suggests that TGF-β1, PDGF-AB and IL-1β are potent stimuli of lung fibroblasts. Pirfenidone did not inhibit the responses to TGF-β1, PDGF-AB and IL-1β, whereas nintedanib demonstrated a significant inhibitory effect on PAI-1 release and cell proliferation but not IL-6-induced release.

4.6. References

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5. Chapter 5 – Discussion

5.1. General Discussion

The hypothesis of this study was that lncRNAs regulate the function of fibroblasts in normal and IPF human lungs. The overall results of this study support the hypothesis as several lncRNAs were identified to be differentially expressed in the IPF lungs as well as in response to pro-fibrotic and pro-inflammatory activation *in vitro*. Additionally, the functional roles of 4 lncRNAs were confirmed using knockdown studies and were found to regulate the activity of human lung fibroblasts.

Prior to studying lncRNA expression and function, the initial stages of this project focused on the pharmacological characterisation of the responses to TGF-β1, PDGF-AB and IL-1β. The effect of these agonists on adult primary human lung fibroblasts provided a better understanding of the optimal conditions required for examining changes in mRNA and lncRNA expression in subsequent studies. These experiments also provided valuable information as to the phenotypic differences between control and IPF fibroblasts. Based upon these investigations, we showed that IPF fibroblasts demonstrated a more fibrotic and a less inflammatory and proliferative profile than control fibroblasts.

As demonstrated in previous studies, TGF-β1 is a potent activator of PAI-1 (Kutz et al., 2001; Murakami et al., 2006; Omori et al., 2016; Takakura et al., 2012) and was therefore used as a measure of the fibrotic response in our studies. Upon TGF-β1 stimulation both control and IPF lung fibroblasts presented a significantly elevated release of the pro-fibrotic protein PAI-1. Most importantly, IPF fibroblasts demonstrated significantly higher sensitivity to TGF-β1 concentrations compared to control fibroblasts as demonstrated by their $\log EC_{50}$ values. PAI-1 release was also elevated over time when incubated with 3 ng/ml TGF-β1, a concentration found to effectively activate the fibroblasts used in this study; similar concentrations in the range of 2-5 ng/ml were previously reported by other studies (RAghu et al., 1989; Aschner et al., 2014; Porte and Jenkins, 2014; Omori et al., 2016).

Incubation of lung fibroblasts with various concentrations of PDGF-AB promoted cell proliferation, a finding consistent with previous studies (Hetzel et al., 2005). However, no significant difference was observed between control and IPF fibroblasts in response to PDGF-AB exposure, possibly due to the high variability between cell populations. As these fibroblasts were isolated from different patients, variability in their response to stimuli was anticipated, which is an issue that could potentially be addressed by increasing the number of biological replicates. Lung fibroblasts also demonstrated a time-dependent increase in cell numbers over the 72-hour period following exposure to 100 ng/ml PDGF-AB. Similar concentrations of PDGF (50-120 ng/ml) have previously been shown to increase the proliferative activity of various cell types including fibroblasts (Makarov et al., 2013; Wollin et al., 2014) whilst other studies have demonstrated an effect at lower concentrations (Hetzel et al., 2005; Vij et al., 2008). Interestingly, unstimulated control fibroblasts isolated from healthy lungs demonstrated significantly higher proliferative capacity compared to unstimulated IPF fibroblasts. This observation is in line with previous findings (Ramos et al., 2001; Hetzel et al., 2005), although other studies reported that IPF fibroblasts proliferate significantly faster (Jordana et al., 1988; Marudamuthu et al., 2015). The slower growth rate of IPF fibroblasts may be explained by their increased basal rate of spontaneous apoptosis as reported by Ramos *et al.* (Ramos et al., 2001). However, this may raise more questions regarding the activity and fate of IPF fibroblasts during the disease as well as the relation between increased programmed cell death and fibroblastic foci formation in the scarred lung.

IL-1β (1-10 ng/ml) was previously shown to promote IL-6 expression in orbital and synovial fibroblasts *in vitro* (Chen et al., 2005; Miyazawa et al., 1998), however no studies have focused on its effect on IL-6 in lung fibroblasts. Exposure to IL-1β induced high levels of IL-6 cytokine release in both control and IPF lung fibroblasts in a concentration-dependent manner. However, in contrast to the TGF-β1 response, control fibroblasts exhibited significantly higher sensitivity to IL-1β treatment compared to IPF fibroblasts, indicating a lower inflammatory profile for IPF fibroblasts. A review of transcriptome

studies by Plantier *et al.* also showed repression of inflammatory and immune pathways in IPF fibroblasts (Plantier et al., 2016). Examination of the time course indicated that IL-6 levels were also considerably elevated in both unstimulated and IL-1β-activated control fibroblasts compared to the corresponding time points in IPF fibroblasts. This finding differs from a recent study showing no significant difference in basal IL-6 levels between control and IPF fibroblasts (Schuliga et al., 2017).

The effects of the IPF drugs nintedanib and pirfenidone on TGF-β1, PDGF-AB and IL-1β-activated lung fibroblasts were also investigated. Pirfenidone elicited no effect on the activity of control or IPF fibroblasts; while nintedanib inhibited the TGF-β1-induced fibrotic response and PDGF-AB-induced proliferative response but not the IL-1β-induced inflammatory response. Interestingly, phenotypic differences between control and IPF fibroblasts were also demonstrated in these experiments, where nintedanib was found to have a significant inhibitory effect on IPF fibroblasts at lower concentrations, both in reducing PAI-1 levels and cell proliferation compared to the controls. However, although pirfenidone did not reduce proliferation or PAI-1 and IL-6 release, this does not reflect its inability to inhibit other IPF-related events. Indeed, pirfenidone was previously shown to attenuate the fibrotic response by reducing proliferation as well as α-SMA and pro-collagen mRNA and protein levels in lung fibroblasts (Conte et al., 2014).

The phenotypic differences between control and IPF lung fibroblasts discussed above were also reflected at the epigenetic level. Using a chromatin immunoprecipitation (ChIP)–specific antibody for the histone modification H3K4me1 in combination with next-generation high-throughput sequencing, we examined the genome-wide distribution profile of H3K4me1 in unstimulated control and IPF lung fibroblasts. The histone modification H3K4me1 is a marker of ‘poised’ promoter and enhancer regions and it is therefore considered as a ‘window of opportunity’ for transcription (Calo and Wysocka, 2013). A clear distinction was observed between the control and IPF lung fibroblasts using unsupervised hierarchical clustering, indicating a difference in the basal epigenetic state of the two cell groups which might

explain their different responses to stimuli such as TGF-β1 and IL-1β, as well as their basal proliferative activity. This is the first genome-wide study to investigate the profile of H3K4me1-enriched DNA regions in IPF fibroblasts or indeed any other histone markers, although previous epigenetic studies showed altered DNA methylation in IPF lung tissues (Sanders et al., 2012) and IPF fibroblasts (Huang et al., 2014).

Upon establishing the phenotypic and epigenetic differences between control and IPF lung fibroblasts, the role of lncRNAs in the regulation of IPF was investigated. As novel mediators of gene regulation, the expression of lncRNAs has not been explored previously in IPF lung fibroblasts; therefore, investigating their expression and ultimately their role in control and IPF fibroblasts was the primary aim of this study. Having characterised the different phenotypic and epigenetic profiles of the two cell populations in the prior experiments, it was interesting to investigate whether these might be attributed to the expression of lncRNAs.

The transcriptomic profiles of non-stimulated and TGF-β1–stimulated control and IPF fibroblasts were investigated using the Affymetrix GeneChip™ Human Transcriptome Arrays 2.0. This is the highest resolution microarray for gene expression profiling with over 6 million probe sets covering both protein and non-protein coding transcripts. The last and only gene profiling study which focused on investigating gene expression in ‘fibrotic’ fibroblasts in response to TGF-β1 exposure was completed more than 10 years ago (Renzoni et al., 2004). However, this study used a much older version of microarray technology and did not examine changes in lncRNA expression. Also notable is that this study did not use only IPF fibroblasts but rather a combination of IPF and scleroderma-associated fibroblasts which were referred to as ‘fibrotic’ fibroblasts. In our study, TGF-β1 activation stimulated the expression of several pro-fibrotic genes in both the control and IPF fibroblasts, however there was not a significant difference in the expression of either mRNAs or lncRNAs between the two groups at the transcriptomic level. This is consistent with the findings of Renzoni *et al.* (Renzoni et al., 2004), where no substantial difference was observed between control and fibrotic

fibroblasts in response to TGF-β1. Hence, the profiles of non-stimulated control and IPF fibroblasts were also compared. Two lncRNAs namely LINC00960 and LINC01140 were identified to be significantly differentially expressed in IPF fibroblasts and their elevated expression was confirmed by qRT-PCR. Notably, analysis of published RNA-seq data from biopsy samples showed that LINC00960 and LINC01140 were expressed in both control and IPF lung tissue, although only LINC01140 demonstrated significant upregulation in the IPF lung. The reasons for these differences are unclear although they may be attributed to the fact that lung biopsies contain a mixture of cells not just fibroblasts, and that lncRNAs expression is typically cell-specific. However, the expression of both lncRNAs in lung tissue provided further evidence that they are present in human lungs and may have an important role in IPF.

The role of LINC00960 and LINC01140 in control and IPF fibroblasts was investigated using knockdown studies. Very little is known about these lncRNAs and nothing is reported regarding their functional roles, with the exception of just one study which has identified LINC01140 as a biomarker for the prognosis of gastric cancer (Song et al., 2017). LINC00960 and LINC01140 knockdown failed to affect the TGF-β1-induced PAI-1 response, However, LINC00960 and LINC01140 were shown to be positive regulators of proliferation both in the presence and absence of PDGF-AB activation. This finding may not be surprising as several other lncRNAs were previously found to regulate cell proliferation in various cell types (Wang et al., 2016; Shao et al., 2017; Park et al., 2018) as well as fibroblasts (Beermann et al., 2018). LINC01140 was also found to be a negative regulator of the inflammatory response as its knockdown significantly increased IL-6 expression and release, particularly in IPF lung fibroblasts. This observation may explain how the elevated expression of LINC01140 in IPF fibroblasts leads to the reduced inflammatory profile, when compared to controls.

The transcriptomic profile of control lung fibroblasts in the presence and absence of the potent pro-inflammatory cytokine IL-1β was also examined for the first time. RNA-seq revealed differential expression of several mRNAs and

lncRNAs, particularly genes associated with inflammatory pathways. Amongst these genes, expression of the lncRNAs IL7AS and MIR3142HG exhibited the highest upregulation and their function was investigated further in subsequent experiments. IL7AS was found to regulate IL-6 release in both control and IPF fibroblasts, a finding similar to previous studies demonstrating the regulatory activity of this lncRNA in the inflammatory response (Pearson et al., 2016; Roux et al., 2017). MIR3142HG knockdown attenuated IL-8 and CCL2 protein release in control fibroblasts, thus demonstrating a positive regulatory role in the inflammatory response. MIR3142HG is a host gene for miR-146a and therefore its knockdown may inevitably reduce the expression of this miRNA. Interestingly, previous studies have found that expression of miR-146a exerts an anti-inflammatory effect and is therefore considered a negative regulator of the inflammatory response (Meisgen et al., 2014; Bhatt et al., 2016; Roos et al., 2016). Contrastingly, a recent knockout study by Pfeiffer *et al.* showed that miR-146a positively regulates inflammation in endothelial cells (Pfeiffer et al., 2017). The contradictive findings may not be unusual given that lncRNAs/miRNAs are known to exert cell-specific functions; thus, the functions of miR-146a are most likely tightly regulated by its lncRNA host gene. Knockdown of MIR3142HG did not affect the expression of any of the pro-inflammatory proteins measured in IPF fibroblasts which may be attributed to the fact that the expression of MIR3142HG was already reduced compared to controls following IL-1β activation and thus no appreciable difference was observed. The reduced expression of this lncRNA in IPF fibroblasts is another interesting finding which may give an insight into the transcriptional differences between control and IPF fibroblasts. As discussed above, IPF fibroblasts were shown to have a reduced inflammatory profile compared to the controls which were more sensitive to IL-1β activation and released elevated levels of IL-6. Interestingly, MIR3142HG was shown to be a positive regulator of inflammation and its expression was particularly elevated in control fibroblasts, which may raise further questions as to how MIR3142HG regulates the inflammatory response in fibroblasts.

5.2. Future work and directions

The experiments described in this thesis have focused upon examining the role of lncRNAs in both non-stimulated control and IPF lung fibroblasts, as well as both cell types exposed to stimuli that drive phenotypes associated with IPF. However, considering the limitations of the study, these observations have raised further questions which require consideration.

The project characterised the effects of TGF-β1, PDGF-AB and IL-1β on control and IPF lung fibroblasts which are three of several proteins associated with the development of IPF. Future studies might look at other cytokines and mediators that have been implicated in the development of IPF. Moreover, increasing the number of biological replicates would enhance the statistical robustness of all experiments and resolve issues related to the variability between patient responses. In addition, although the principal focus of the project was upon examining the differences in fibrotic response, we also uncovered significant differences in the inflammatory response between control and IPF fibroblasts. The observation that IPF fibroblasts have a reduced inflammatory profile compared to the controls raised key questions regarding the role of inflammation in the development of IPF. Interestingly, previous attempts to treat IPF with immunosuppressants have failed and have even been shown to exacerbate IPF in several cases (Spagnolo et al., 2015a). Although evidence suggests the immune response is suppressed in IPF fibroblasts, it appears that the presence of a prominent immune inflammatory infiltrate in IPF lungs plays a potential role in early disease progression (Balestro et al., 2016). Hence, the role of inflammation and more generally the immune response in IPF is still unclear and largely unexplored. Whether the role of the immune response changes as the disease progresses and how it affects the activation of different cells in human lungs has yet to be explored.

The epigenetic landscape of control and IPF fibroblasts also showed distinct differences as observed by the H3K4me1-enriched DNA regions. However, there was no correlation between the H3K4me1-associated regions and the lncRNAs or mRNAs that were differentially expressed in IPF fibroblasts.

Although H3K4me1 provides insight regarding DNA regions associated with promoters and enhancers that are poised for transcription, future studies might look at other common histone markers such as those for active promoters (H3K4me3) or active transcription (H3K27ac). Epigenetic modifications are gaining increasing interest as therapeutic targets as indicated by the success of histone deacetylase (HDAC) inhibitors and DNMT inhibitors as cancer treatments (Pfister and Ashworth, 2017).

The transcriptomic data in this study revealed hundreds of differentially expressed genes in response to exposure to TGF-β1 and IL-1β, of which four lncRNAs were selected for further investigation. However, although modern array and sequencing technology are incredibly powerful, one of the limitations that should be considered is that they only provide a snapshot into the transcriptional state of the cells at a very specific point in time. As there is a constant turnover of transcripts in a cell reflecting changes in gene expression, this inevitably limits the capacity of sequencing to resolve changes in cellular activity over time. As such, examining the transcriptome of IPF fibroblasts at different time points may yield better insights as to the overall gene expression activity, which would be otherwise overlooked.

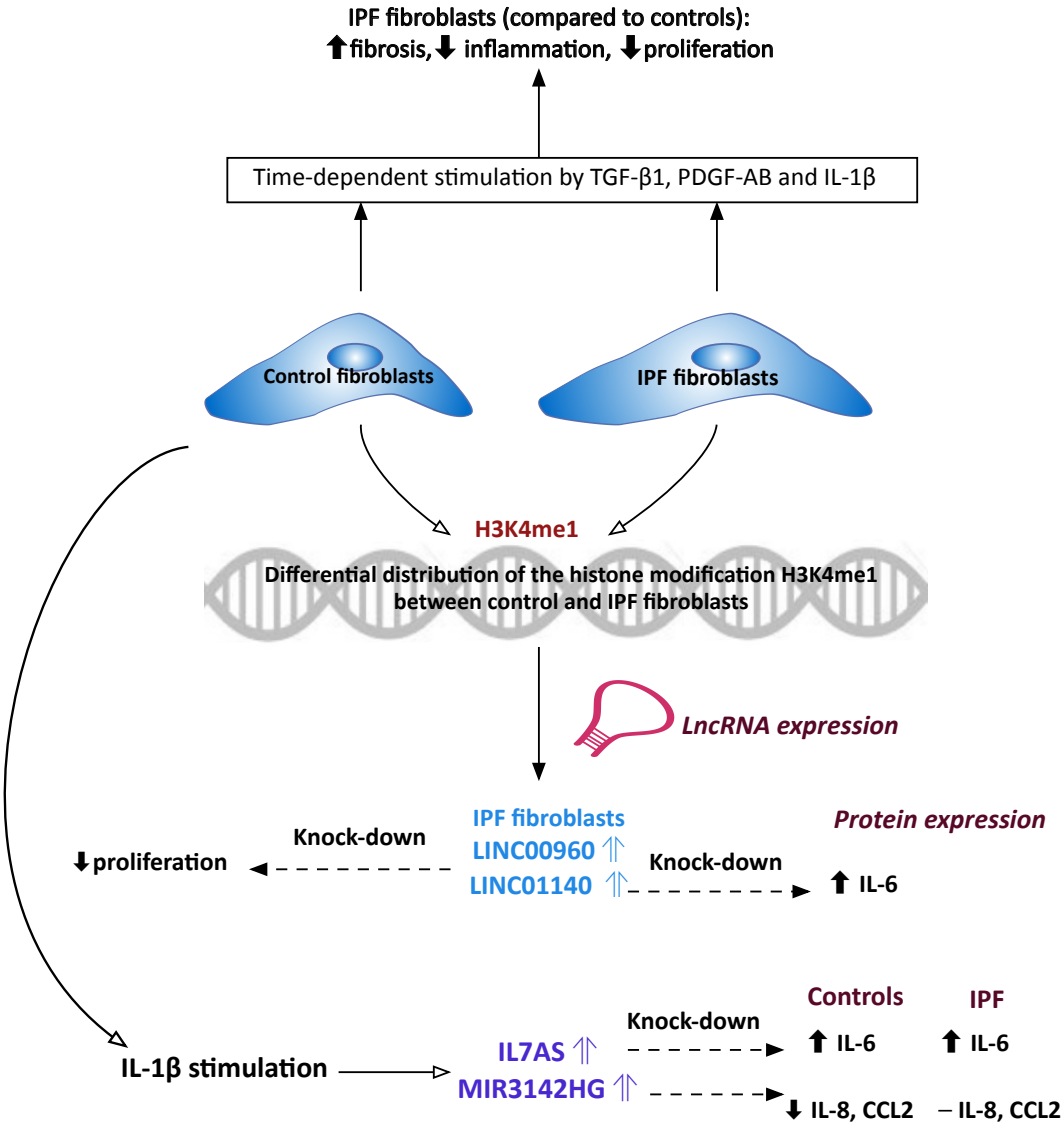
One of the most important aspects of this study were the functional studies around the four differentially expressed lncRNAs. Evidently, more functional and mechanistic studies are essential to further understand the function of these lncRNAs. For example, knockdown of LINC00960 and LINC01140 did not demonstrate a significant effect on the PAI-1 release which was a measure of the fibrotic response. However, it is unknown if they regulate other proteins associated with the fibrotic response which was not possible to examine in this project. Likewise, although LINC01140, IL7AS and MIR3142HG were shown to regulate cytokines of the inflammatory response, only a number of cytokines were investigated and therefore the full effect of these lncRNAs on gene expression is unclear. As such, employing array or sequencing technology on these lncRNA knockdown samples would be of great interest to gain an overview of the effect they have on global gene expression.

Very little is known about these lncRNAs and therefore further functional studies should focus not just on which genes are affected by their expression, but also their mechanism-of-action. With the exception of IL7AS, it is currently unknown whether these lncRNAs are located in the nucleus or the cytoplasm. IL7AS was previously found to be enriched in the nucleus in THP-1 and A549 cell lines (Roux et al., 2017). Nuclear-cytoplasm RNA fractionation could be used to determine the subcellular distribution of the other lncRNAs which would provide some indication as to their potential mechanism. In the longer term, it would be crucial to investigate protein-lncRNA interactions to determine the mechanism by which they function.

Examining isolated fibroblasts *in vitro* enables the investigation of these cells individually, however it is equally important to examine how fibroblasts interact with other cells, as well as under exposure to multiple physiologically relevant signals and stimuli. Investigating the *in vivo* effects of lncRNAs in the bleomycin model of fibrosis may thus generate a useful insight into such effects; however, the poor conservation of lncRNAs between species may present further challenges in this context. One potential means to overcome this would be to examine the expression profile of lncRNAs in cells isolated from lung tissue following dissociation and separation using fluorescence-activated cell sorting (FACS). However, although this approach offers the prospect of more physiologically representative cell populations, the lack of well characterised fibroblast-specific markers hinders reliable sorting of sufficient numbers of fibroblasts for downstream applications.

Finally, as a consequence of their cell/tissue-specific expression, the identification of lncRNAs that drive the activity of IPF lung fibroblasts may present a great opportunity for the development of novel treatment strategies. Several oligonucleotide antisense therapeutics have already proven successful and are being administered to patients, while nucleic acid-targeting drugs have demonstrated great potential in targeting lncRNAs in cancer (Arun et al., 2018). Better delineating the functions of lncRNAs in lung fibroblasts may thus render them as potential targets for pharmacological intervention for IPF.

5.3. Diagram of key findings



- Control and IPF fibroblasts were stimulated in a time-dependent manner in response to TGF-β1, PDGF-AB and IL-1β
- IPF fibroblasts demonstrated a more fibrotic and a less inflammatory and proliferative profile compared to control fibroblasts
- A clear distinction was observed between the H3K4me1-enriched DNA regions of control and IPF fibroblasts
- Differential gene expression of several mRNAs and lncRNAs was observed upon TGF-β1 stimulation of control and IPF fibroblasts
- IPF fibroblasts demonstrated increased expression of the lncRNAs, LINC00960 and LINC01140
- LINC00960 and LINC01140 were shown to be positive regulators of proliferation
- LINC01140 was shown to be a negative regulator of IL-6 expression and protein release, possibly mediating the reduced inflammatory response observed in IPF fibroblasts
- Differential gene expression of several mRNAs and lncRNAs was observed upon IL-1β stimulation of control fibroblasts
- LncRNAs IL7AS and MIR3142HG demonstrated the highest upregulation upon IL-1β exposure in control fibroblasts
- IL7AS was found to regulate IL-6 release in both control and IPF fibroblasts
- MIR3142HG demonstrated a positive regulatory role for IL-8 and CCL2 protein release in control fibroblasts but not IPF fibroblasts
- Nintedanib had an inhibitory effect on PAI-1 release and cell proliferation in control and IPF fibroblasts

5.4. References (Chapter 1 and Chapter 5)

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