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# The Extracellular Matrix Protein Fibulin-1 in Idiopathic Pulmonary Fibrosis

A thesis submitted for the degree of

Doctor of Philosophy

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# Preface

All the experimental work reported in this thesis was carried out by the candidate, except where acknowledgment of the work of others is given in the text. No portion of this work has been submitted by the candidate toward the award of any other degree. Ethical approval for this project was provided by the Human Ethics Committee of the University of Sydney (Australia), Belberry Human Research Ethics Committee (Perth, Australia), Comitato Etico Provinciale di Modena (Modena, Italy) and the Laurel Heights Panel (San Francisco, USA).

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This thesis is dedicated to you all.

# List of Abbreviations

| | |
|---|---|
| AFM | Atomic force microscopy |
| AIHW | Australian Institute of Health and Welfare |
| ANCOVA | Analysis of covariance |
| ANOVA | Analysis of variance |
| APS | Ammonium persulphate |
| a-SMA | Alpha smooth muscle actin |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| COPD | Chronic obstructive pulmonary disease |
| CT | Computed tomography |
| CTD-ILD | Connective tissue disease related interstitial lung disease |
| $DL_{CO}$ | Diffusing capacity of the lung for carbon monoxide |
| $DL_{CO}$% | Percentage predicted diffusing capacity of the lung for carbon monoxide |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiotheritol |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid disodium salt |
| ELISA | Enzyme-linked immunosorbant assay |
| EMT | Epithelial-mesenchymal transition |
| FBLN1 | Fibulin-1 |
| FBS | Foetal bovine serum |
| $FEV_1$ | Forced expiratory volume in 1 second |
| $FEV_1$% | Percentage predicted forced expiratory volume in 1 second |
| FN | Fibronectin |
| FVC | Forced vital capacity |
| FVC% | Percent predicted forced vital capacity |
| HBSS | Hank's balanced salt solution |
| HP | Hypersensitivity Pneumonitis |
| IHC | Immunohistochemistry |
| IIP | Idiopathic interstitial pneumonia |
| IL | Interleukin |

| | |
|---|---|
| ILD | Interstitial lung disease |
| IPF | Idiopathic pulmonary fibrosis |
| kDa | Kilodalton |
| LAM | Lymphangioleiomyomatosis |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger ribonucleic acid |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| Pen-Strep | Penicillin-Streptomycin |
| PFA | Paraformaldehyde |
| PO | Periostin |
| PVDF | Polyvinylidene flouride |
| QPCR | Quantitative polymerase chain reaction |
| RT | Reverse transcription |
| Sarcoid | Sarcoidosis |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error mean |
| TEMED | Tetramethylethylenediamine |
| TGFβ | Transforming growth factor beta |
| TMB | Tetramethylrhodamin iso-thiocyanate |
| TNC | Tenascin-C |
| v/v | Volume to volume ratio |
| w/v | Weight to volume ratio |

# Abstract

One of the most pressing issues in the clinical management of patients with idiopathic pulmonary fibrosis (IPF) is how a clinician can respond when asked “how long do I have?” IPF has a varied clinical course and the only available treatments, aside from lung transplantation, are corticosteroids and immunosuppressants. The side effects of both corticosteroids and immunosuppressants may actually outweigh the benefits for use in IPF. Biomarkers of disease progression are often unable to predict acute lung function decline. This is possibly because the underlying mechanisms driving the disease are poorly understood and little attention has been paid to how intrinsic differences in resident lung fibroblasts may be contributing to this disease.

In this thesis, extracellular matrix (ECM) molecules that are both found in the blood and released by resident lung fibroblasts were investigated for their utility as biomarkers of disease progression in IPF. The primary focus was on the ECM protein fibulin-1, an essential constituent of elastic fibres, which has not previously been studied in the context of interstitial lung disease. This thesis investigated the relationship between fibulin-1, and disease severity in patients with and without pulmonary fibrosis. In addition, the utility of fibulin-1 as a biomarker of disease progression was compared against other previously described components of the ECM, namely periostin, tenascin-C and fibronectin, in the same patients. Lastly, the effect of the profibrotic cytokine transforming growth factor-beta-1 (TGFβ1) on fibulin-1 levels in resident lung fibroblasts from patients with and without IPF was interrogated.

Patients with IPF had higher levels of serum, tissue and fibroblast-derived fibulin-1 than patients without IPF. Serum fibulin-1 levels accurately discriminated between patients with IPF who progressed (whereby progression is defined as a decline in lung function) within one year of blood draw and those who remained stable. Neither periostin, tenascin-C or fibronectin levels were predictive of disease progression in patients with IPF.

Increased ECM deposition leads to decreased lung function as the normal architecture is replaced by fibrotic tissue. Tissue fibulin-1 negatively correlated with lung function in patients with IPF. Importantly, while tissue periostin and total collagen were similarly increased in patients with IPF compared to patients without IPF, levels of periostin and total collagen did not correlate with lung function. This thesis highlights for the first time that fibulin-1 may be an important contributor to lung mechanical properties. Furthermore, fibulin-1 dysregulation may stem from altered fibulin-1 production by fibroblasts derived from patients with IPF. Cell-secreted and cell-associated fibulin-1 levels were increased in fibroblasts derived from patients with IPF compared to patients without IPF. TGFβ1 induced deposition of fibulin-1 was observed in fibroblasts derived from patients with IPF but not in fibroblasts derived from patients without IPF.

In conclusion, the ECM profile is altered in patients with IPF compared to patients without IPF. While any of the 300+ proteins that constitute the lung ECM may be dysregulated in the context of IPF, fibulin-1 stood out as a novel biomarker of disease severity and may be an important target of the fibrotic process.

# Publications arising from this thesis

## Published journal manuscripts

Fibulin-1 predicts disease progression in patients with pulmonary fibrosis
***Jaffar J,*** *Unger S, Corte TJ, Keller M, Wolters PJ, Richeldi L, S Cerri S, Prele CM, Hansbro PM, Argraves WS, Oliver BGG, Oliver RA, Black JL and Burgess JK*
*Chest. 2014 May 15. doi: 10.1378/chest.13-2688*

## Manuscripts in preparation

Transforming growth factor-beta 1 increases fibulin-1 in fibroblasts from patients with idiopathic pulmonary fibrosis

Primary parenchymal fibroblasts from patients with idiopathic pulmonary fibrosis are stiffer than fibroblasts from patients without idiopathic pulmonary fibrosis as measured by atomic force microscopy.

## Published conference abstracts

## International

The Matricellular Protein Fibulin-1 Is Increased In Primary Parenchymal Fibroblasts Derived From Patients With Idiopathic Pulmonary Fibrosis
***Jaffar J,*** *Oliver BGG, Black JL, Burgess JK*
*American Thoracic Society International Conference Abstracts 2014, San Diego*
*Volume 269, A6647*
*DOI: 10.1164/ajrccm-conference.2014.189.1_MeetingAbstracts.A6647*

Fibulin-1 is a novel biomarker of disease severity in pulmonary fibrosis
*Burgess JK,* ***Jaffar J****, S Unger, Keller M, Corte TJ, Wolters PJ, Richeldi L, Cerri S, Argraves WS, Black JL, Oliver BGG*
*European Respiratory Society Conference 2013, Barcelona*
*Volume 42, Suppl. 57, p. 69S*
*First author must present at this conference. Supervisor Burgess presents on behalf of Jaffar J.*

Levels of fibulin-1 in the lung and serum are increased in fibrotic interstitial lung disease
***Jaffar J,*** *Unger S, Corte TJ, Wolters PJ, Richeldi L, Cerri S, Argraves WS, Oliver BGG, Black JL, Burgess JK*
*American Thoracic Society International Conference Abstracts 2013, Phildelphia*
*Volume 272, A3382*
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Is the extracellular matrix protein fibulin-1 a key player in idiopathic pulmonary fibrosis?
*Burgess JK,* ***Jaffar J****, Oliver BGG, Corte TJ, Argraves WS, Twal WO, Wolters PJ, and Black JL.*
*International Colloquium on Lung and Airway Fibrosis 2012 Conference, Modena*

Does the dysregulation of the ECM result in the pathology of Lymphangioleiomyomatosis?
*Burgess JK,* ***Jaffar J****, Tjin G, Weckmann M, Heckman CA, Corte TJ, Argraves WS, Twal WO, Moir LM, Black JL, Oliver BGG*
*International Colloquium on Lung and Airway Fibrosis 2012 Conference, Modena*

The release of soluble fibulin-1 from airway epithelial cells is increased by transforming growth factor-beta
***Jaffar J****, Tan X, Black JL, Oliver BGG, Argraves WS, Twal WO, Burgess JK*
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The serum level of Fibulin-1 is elevated in idiopathic pulmonary fibrosis.
***Jaffar J****, Tan X, Black JL, Oliver BGG, Corte T, Wolters PJ, Argraves WS, Twal WO, Burgess JK*
*American Thoracic Society International Conference Abstracts 2012, San Francisco*
*Volume 315, A5173*
*DOI:10.1164/ajrccm-conference.2012.185.1_MeetingAbstracts.A5173*

## Local

The Matricellular Protein Fibulin-1 is a Better Marker of Disease Progression than Periostin, Tenascin-C and Fibronectin In Patients with Idiopathic Pulmonary Fibrosis
***Jaffar J****, Van Ly D, Munk L, Corte TJ, Cerri S, Richeldi L, Wolters PJ, Prele CM, Oliver RA, Oliver BGG, Black JL, Burgess JK*
*Respirology 2014, Volume 19, Issue S2, p. 32–34,*

Primary Lung Fibroblasts from Patients with IPF Show Increased Stiffness which may be due to Differential Production of ECM Proteins
***Jaffar J,*** *Chrzanowski W, Faiz A, Wolters PJ, Oliver BGG , Black JL, Burgess JK.*
*Respirology 2014, Volume 19, Issue S2, p.18-20*

The extracellular matrix protein fibulin-1 is increased in patients with IPF
***Jaffar J****, Tjin G, Unger S, Black JL, Oliver BGG, Burgess JK*
*Respirology 2013, Volume 18, Issue S2, p.27*

Fibulin-1 and periostin levels are associated with the severity of fibrotic lung disease
*Keller M and* ***Jaffar J,*** *Corte TJ, Black JL, Webster S, Troy L, Corte P, Torzillo P, Burgess JK*
*Respirology 2013, Volume 18, Issue S2, p. 47*

Transforming growth factor-beta increases the release of soluble fibulin-1 in primary airway epithelial cells.
***Jaffar J****, Tan X, Black JL, Oliver BGG, Argraves WS, Twal WO, Burgess JK*
*Respirology 2012, Volume 17, Issue S1, p.P-003*

Fibulin-1 is increased in the blood of idiopathic pulmonary fibrosis patients.
***Jaffar J****, Tan X, Black JL, Oliver BGG, Corte T, Argraves WS, Twal WO, Wolters P, Burgess JK*
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## Awards linked to abstracts

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- Thoracic Society of Australia and New Zealand Travel Award 2012
- Woolcock Institute of Medical Research Symposium Award (Overall best presentation) 2013

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# Chapter 1. **Introduction**

## 1.1 Interstitial lung disease

### 1.1.1 Background

The term interstitial lung disease (ILD) is an umbrella term that describes a large group of disorders that can affect men, women and children (Bradley et al. 2008). Most of the more than 150 different ILD disorders eventually lead to lung scarring, or pulmonary fibrosis (Selman and Pardo 2013). Causes of ILDs are varied and often hard to determine in the individual patient. Some patients could go many years without a definitive diagnosis and disease guidelines outline the need for a multidisciplinary approach for the most accurate diagnosis (Raghu et al. 2011).

Diagnosis of ILD is challenging and there is an entire subgroup of ILDs of which the cause of the initial injury is ruled unknown. Idiopathic interstitial pneumonias (IIPs) encompass an equally varied spectrum of ILDs in which all typical causes of ILD have been ruled out, which puts a special emphasis on the need for a complete patient history in addition to baseline lung function measurements during the initial presentation. However, pulmonary fibrosis remains a common theme and a cause for concern in many ILDs.

In pulmonary fibrosis, the alveoli that are responsible for efficient gas transfer are hindered by perpetual inflammation and damaged interstitium. When scarring occurs, it is generally not reversible (Paz and Shoenfeld 2010). Progressive fibrosis eventually leads to the destruction of the normal lung architecture, loss of lung function and eventually death (Araya and Nishimura 2010).

Changes in lung function parameters are often used to monitor patients with ILD. In patients with idiopathic pulmonary fibrosis (IPF), a small decline in lung function can indicate increased risk of death due to fibrosis. Pulmonary fibrosis is a consequence for which lung transplantation remains the only viable option of treatment in the end stages of different ILDs (Demedts et al. 2001; Raghu et al. 2011).

Greater understanding of the mechanisms driving pulmonary fibrosis is critical in order to design treatments to slow or even reverse the fibrotic process. The extracellular matrix (ECM) is a key driver of the fibrogenesis (Tschumperlin et al. 2012). Changes in ECM proteins signal states of active fibrogenesis in other diseases (Jourdan-LeSaux et al. 2010; Lopez-Hernandez and Lopez-Novoa 2012) and have recently also been shown to be important in IPF (Naik et al. 2012).

### 1.1.2 Aetiology

There are many different causes of ILD and identification of the causal agent is an essential component of the diagnostic strategy. Classification of ILD is based on aetiology (Figure 1.1) and the clinical course of ILD is largely determined by its underlying cause. Most commonly, ILDs will be associated with occupational or environmental exposures (eg. smoke, bird allergens) or due to an underlying connective-tissue disease (eg. scleroderma).

Although some forms of ILD are untreatable, many forms do respond to treatment (Theodore et al. 2012). This makes accurate diagnosis of the specific ILD very important. ILDs can be grouped into categories where the initial insult is known, and those where the cause of the ILD is unknown, or idiopathic.

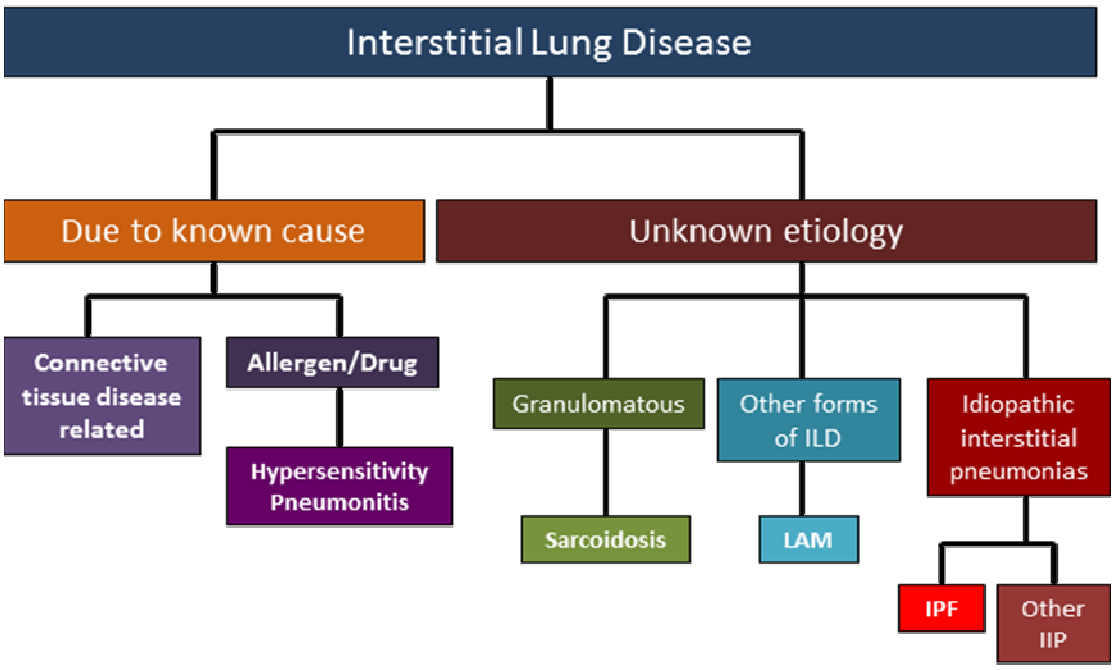



**Figure 1.1 Classification of some Interstitial Lung Diseases (ILDs)**
The classification of ILDs is based primarily by aetiology.
IIP idiopathic interstitial pneumonia, IPF idiopathic pulmonary fibrosis, LAM lymphangioleiomyomatosis

### 1.1.3 Diagnosis

Diagnosis in ILD is difficult as the scores of disease entities all affect the lung interstitium and share similar clinical and radiological manifestations. Prior to diagnosis, a patient will often present with very diffuse respiratory symptoms (Kameda et al. 2011). These symptoms include shortness of breath (dyspnea) and a persistent but non-productive cough. Upon taking a patient history, the clinician will need to determine if there have been any exposures to agents of ILD such as a history of smoking and even certain medications. The clinician also needs to investigate if the ILD is caused by an underlying connective-tissue disease.

A thorough patient history is critical to identify the causal factor and if a patient is suspected to have an ILD, a detailed physical examination follows, again looking for potential underlying causes of ILD not easily elucidated from a patient's memory. Lastly, a series of lung function tests and high-resolution computed tomography (HR-CT) scans will be performed; not only for diagnosis, but also to assess the severity of the ILD at first presentation, as this is indicative of mortality in certain ILDs (Jegal et al. 2005).

If a definitive diagnosis cannot be made following the routine examination, a surgical lung biopsy is then taken. However, this is an invasive procedure and it is possible that diagnosis can be made on the radiological features on HR-CT instead (Ryerson and Collard 2013).

### 1.1.4 Treatment and Management

Management in ILD is multifaceted. Often, for ILDs which have a known aetiology, correct identification of the causal agent is required at first consultation. Patients with

newly diagnosed ILD are all advised to cease smoking and undergo pulmonary rehabilitation which can improve or prevent the further decline of lung function. Monitoring of ILD patients is done through repeated lung function measurements.

In most cases, if the causal agent is an allergen or environmental toxin, removal or limitation of exposure to the agent is recommended. If the causal agent is an underlying connective tissue disease, then the connective tissue disease is treated with the paramount aim of slowing of lung function decline. However, some instances of ILD, such as IPF, are relentlessly progressive and currently have no effective treatment beyond lung transplantation.

### 1.1.5 Connective-tissue disease related interstitial lung disease

Connective-tissue disease related ILD (CTD-ILD) accounts for about 15% of all ILDs (Fischer and du Bois 2012). Underlying connective tissue disease represents a difficult-to-treat sub-group of ILDs, including rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis. As with other ILDs, there is a lot of heterogeneity between CTD-ILDs and each is associated with particular clinical features.

Most clinically relevant is the fact that CTD-ILD often has a more favourable prognosis compared to the other idiopathic ILDs. There have been substantial gains in the understanding of systemic sclerosis through the recent completion of two major clinical trials (Homer and Herzog 2010). Features of CTD are often seen in patients diagnosed with ILD as 13% of those meeting the diagnostic criteria for IPF also have undifferentiated CTD (Fischer and du Bois 2012).

### 1.1.6 Hypersensitivity pneumonitis

Hypersensitivity pneumonitis (HP) is similar in presentation to IPF but is caused by repeated exposure to a particular allergen. Inhalation of organic allergens is the most common cause, but inorganic chemicals can also cause HP (Hanak et al. 2007). Treatment mostly requires avoidance of the causal antigen. HP comes in three states; acute, subacute and chronic, and the majority of patients will present with a pattern that fits one of the three categories (Bradley et al. 2008). Despite this, patients with chronic HP are the most difficult to diagnose as careful patient history must be obtained to rule out IPF (Selman and Buendia-Roldan 2012).

### 1.1.7 Sarcoidosis

Sarcoidosis is an ILD that rarely progresses to pulmonary fibrosis and 55-90% of patients with stage I disease will undergo spontaneous remission (Bradley et al. 2008). Again, the natural history and progression of this disease is difficult to predict but most remissions will occur during the first six months. Due to the high rates of remission, treatment is not normally prescribed if the patient has normal or mildly abnormal lung function and normal oxygen saturation (Reich 2012). If the disease progresses, the overall mortality is 1-5%, mainly due to myocardial or central nervous system involvement (Bradley et al. 2008).

### 1.1.8 Idiopathic interstitial pneumonias

The IIPs includes seven clinico-radiologic-pathologic groups of ILDs: IPF, nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP), acute interstitial pneumonia (AIP), respiratory bronchiolitis-associated interstitial lung

disease (RB-ILD), desquamative interstitial pneumonia (DIP), and lymphoid interstitial pneumonia (LIP).

The availability of HR-CT allowed for the discrimination of a particular radiologic pattern known as usual interstitial pneumonia (UIP) from the other IIP patterns. UIP is the hallmark feature of IPF (Figure 1.2), with its presence determining IPF diagnosis with the greatest certainty (King et al. 2001). Of the seven disease entities, IPF remains the most common of the IIPs and has the greatest mortality (Flaherty et al. 2003).

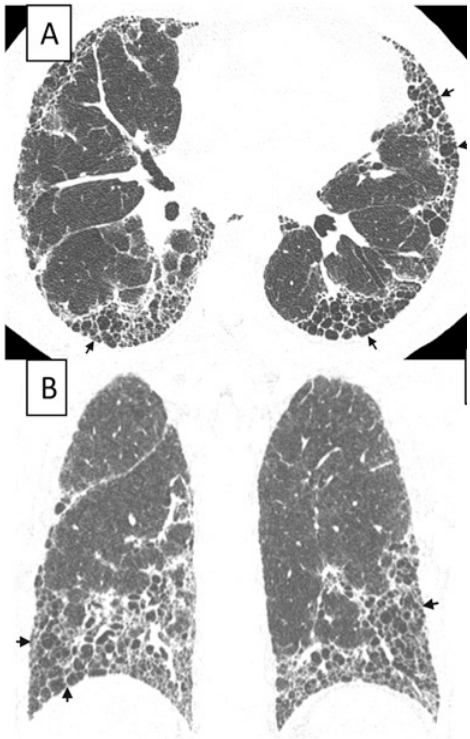



**Figure 1.2 High-resolution computed tomography (HR-CT) scans of a patient with usual interstitial pneumonia (UIP).**
HR-CT scans demonstrate the classic UIP pattern that defines idiopathic pulmonary fibrosis (IPF). (A) Axial and (B) coronal images show extensive fibrosis with basal predominance of the honeycombing pattern that is the hallmark feature of UIP (*arrows*).
Reproduced with permission from (Raghu et al. 2011)

## 1.2 Idiopathic pulmonary fibrosis

### 1.2.1 The problem of an unknown cause

IPF occurs primarily in older adults and fibrosis is limited to the lungs. The diagnosis of IPF is determined by the *exclusion* of other known causes of ILD and a particular pattern of fibrosis that is seen on HR-CT scans or by surgical biopsy (Raghu et al. 2011). In addition, many patients with IPF present with concomitant diseases such as pulmonary hypertension and emphysema, making both diagnosis and treatment difficult.

IPF affects more males than females and smoking is a significant risk-factor for the development of the disease (Ryerson and Collard 2013). Progression in IPF is varied and unpredictable and the effect of co-morbidities has not been well-studied. Unlike other ILDs where allergen avoidance or medications can significantly improve lung function, a diagnosis of IPF has no such positives and clinicians and patients alike are often at a loss as to what are the next steps to take (Swigris et al. 2005).

Current recommendations for treatment are limited to only oxygen therapy in the most severe patients (Raghu et al. 2011). Enrolment into clinical trials may be the only course of action, besides lung transplantation, but often strict inclusion criteria leave many patients without options.

### 1.2.2 Clinical trials

Treatment of IPF has been largely unsuccessful (Noble et al. 2011), partly due to incomplete knowledge of the mechanisms driving fibrogenesis. Pirfenidone ((5-methyl-1-phenyl-2-[1H]-pyridone; Shionogi & Co., Ltd., Osaka, Japan; MARNAC

Inc., Dallas, TX, USA) is a synthetic molecule that acts on transforming growth factor-beta 1 (TGFβ1) and tumour necrosis factor (TNF)-α *in vitro* (Raghu et al. 1999), cytokines thought to play major roles in the development of fibrosis (Leask and Abraham '2004).

Phase 3 results from one of the most recent trials in IPF, the CAPACITY (Clinical Studies Assessing Pirfenidone in idiopathic pulmonary fibrosis: Research of Efficacy and Safety Outcomes) programme, have just been published (King et al. 2014) and aimed to confirm the results of Phase 2 trials of the drug in patients with IPF. Previous studies in a Japanese cohort of patients with IPF indicated that pirfenidone was successful at increasing the progression-free survival time and improved forced vital capacity (FVC) in 163 patients compared to 104 patients who were in the placebo group (Taniguchi et al. 2011).

In a bleomycin model of mouse fibrosis, pirfenidone suppresses the induced elevation of lung basic-fibroblast growth factor and prevents the downregulation of interferon-γ (Oku et al. 2008). It was hypothesized that by targeting the cytokines that were major drivers of the fibrotic environment that the development of chronic pulmonary failure could be slowed. However, in the CAPACITY study, they found that while overall there was a favourable outcome for the use of pirfenidone as a treatment in IPF, a substantial number of patients experienced significant side-effects including nausea, photosensitivity and rash, to name a few (Noble et al. 2011). Furthermore, the greatest benefits of the drug, an increase in percentage predicted forced vital capacity (FVC%), was no longer significant at 72 weeks.

One interpretation of this finding is that while able to suppress the acute pro-fibrotic effects of cytokines, which are largely non-specific to fibrosis, pirfenidone does not

act on the underlying mechanism that specifically drives excessive ECM deposition in the lung interstitium. Therefore, a greater understanding of what distinctively induces ECM deposition is needed and biomarkers of disease progression that reflect states of active lung fibrogenesis would be ideal targets for drug development in IPF.

### 1.2.3 Biomarkers of progression

Peripheral blood biomarkers of disease progression in IPF have been identified and are the most likely to achieve clinical utility because of their ease of sampling (Vij and Noth 2012). A biomarker of disease progression could tie the physical development of fibrosis (ie. excessive ECM deposition) with the cell type most responsible for the production of matrix components, the resident fibroblast.

Of the known serum biomarkers in IPF, most are produced by alveolar epithelial cells or circulating immune cells (Richards et al. 2012; Vij and Noth 2012). Those that are associated with mucus producing epithelial cells include the MUC5B gene polymorphism (Seibold et al. 2011), mucin-1 (KL-6), intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1), surfactant proteins SP-A and SP-D and periostin (Naik et al. 2012). Matrix metalloproteinases 1 and 7, chemokines CCL18 and CXCL8, calgranulin B (S100A12) are produced by macrophages and other immune cells (Rosas et al. 2008; Vij and Noth 2012).

Of these potential biomarkers, only periostin is a matricellular protein and its production is predominantly linked to the epithelium (Sidhu et al. 2010). However, as fibroblasts are the main producers of ECM (Kisseleva and Brenner 2008), more biomarkers related to activated resident lung fibroblasts are needed.

### 1.2.4 ECM proteins as biomarkers in IPF

The development of fibrosis is by definition the dysregulation of ECM production resulting in net deposition by lung fibroblasts. The ECM is created by secretion of ECM components and their extracellular organization into a distinct structure by mechanisms that remain to be fully elucidated. Particular ECM components are also found as serum proteins that are released from the ECM by matrix metalloproteinases (MMPs) (Mott and Werb 2004) or other endogenous enzymes.

Changes in the levels of serum ECM proteins have been used to identify states of disease in cancer (Liu et al. 2006), infertility (Liu et al. 2011), and diabetes (Cangemi et al. 2011) that reflect their respective changes in ECM. In addition, the matrix is bioactive (Roman and Mcdonald 1993), influencing the behaviours of the cells that inhabit it (Perumpanani et al. 1998; Frantz et al. 2010). Therefore, it has been hypothesized that ECM proteins found in the blood, like periostin, have potential as biomarkers of activated fibroblasts and therefore disease progression in IPF (Vij and Noth 2012).

## 1.3 The extracellular matrix

### 1.3.1 Conservation throughout evolution

The ECM is made up of hundreds of different proteins each with a different purpose (Hynes 2012). Many of these proteins are highly conserved throughout the animal kingdom (Hynes 2012). The reason the diversity of ECM molecules was able to evolve is due to the modular structure of their protein domains. This layout allows for extensive exon shuffling during the molecular course of evolution (Engel 1996). The EGF-like repeats that are found as a common feature of ECM proteins can mediate

protein-protein interactions (Kubota et al. 2004). The emergence of extracellular proteins allowed for the physical linking of individual cells, allowing for the basis of the development of multicellular organisms (Frantz et al. 2010).

The general structure of the ECM is similar throughout the body (Figure 1.3) but organ specific changes in ECM composition result in different biochemical and biophysical profiles (Bosman and Stamenkovic 2003). The conceptual understanding of the function of the ECM started out as a simple physical barrier between different cell types (Vracko 1974) but knowledge of its influence on cellular behaviour is increasing (Pardo and Selman 2001; Marinkovic et al. 2012; Parker et al. 2014).

Even though the ECM is fundamentally composed of water, proteins and polysaccharides, each tissue has a specific combination of these components in which a dynamic cross-talking relationship between cells and their extracellular environment is made possible. Maintenance of the ECM is regulated through selective deposition and degradation of ECM proteins via MMPs and tissue inhibitors of metalloproteinases (TIMPs)(Nagase et al. 2006) and other endogenous enzymes such as cathepsins (Xie et al. 2008).

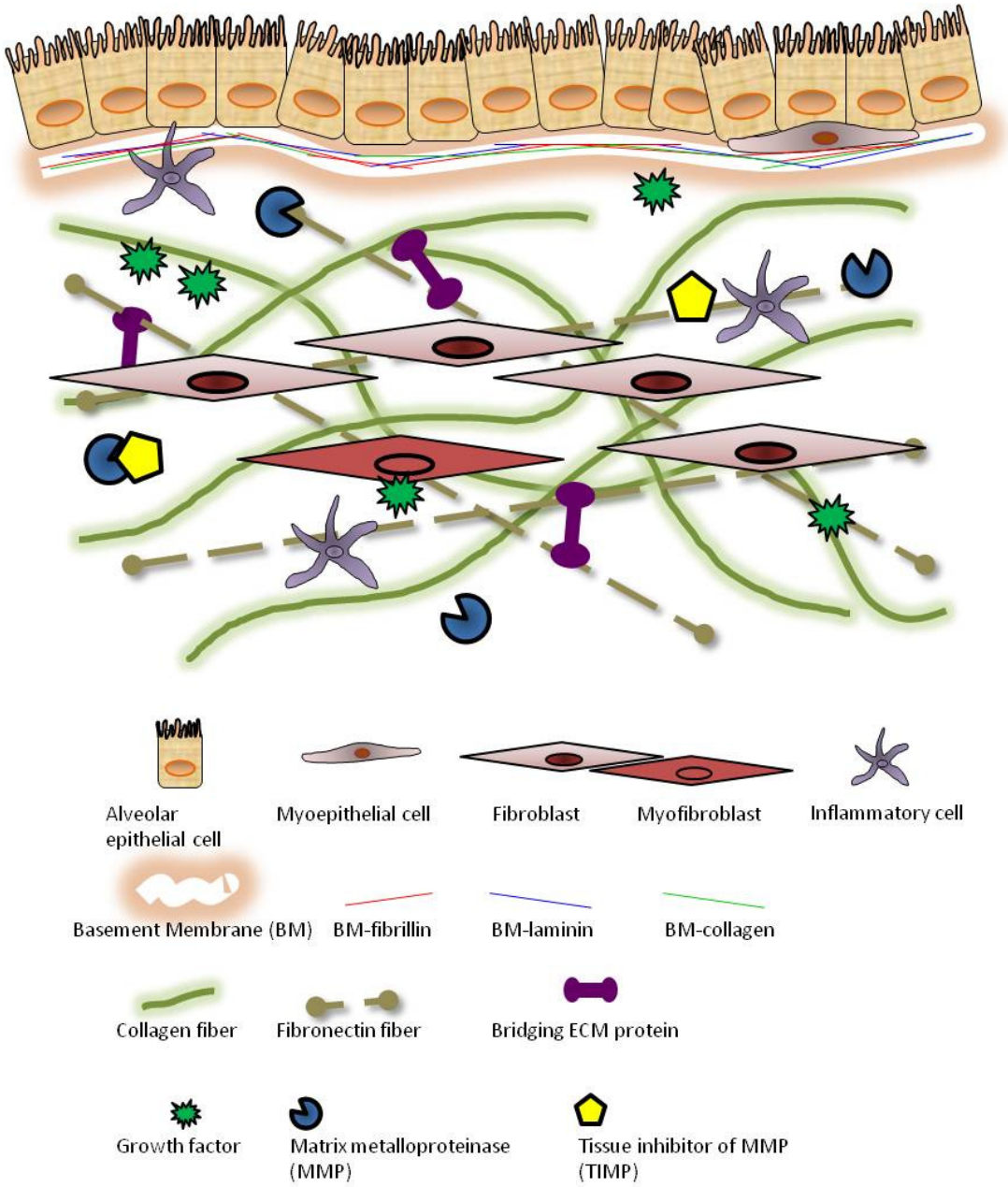



**Figure 1.3 Diagrammatic representation of the general structure of the extracellular matrix (ECM) in the lung parenchyma**
The ECM supports many cells and is made of proteins that each have a role in the maintenance of the interstitial space of the parenchyma.
The interstitum contains many cells and is made of ECM proteins and a diversity of cellular elements.

The ECM is not simply an inert scaffold but also contributes to essential parts of homeostasis, including cell-to-cell signalling (Perumpanani et al. 1998) and cytokine regulation (Crosby and Waters 2010). The ECM interacts with many cell types and provides an essential chemical and physical framework for intrabody communication.

### 1.3.2 A bioactive entity

The control of cell proliferation (Krimmer et al. 2012), differentiation (Maxson et al. 2012), migration (Kubota et al. 2006) and other biological processes is directly and indirectly driven by the ECM and it is particular constituent proteins that directly affect cell behaviour (Hynes 2009; Royce et al. 2009). ECM proteins do not only have biological functions *in situ*, as soluble fragments of ECM have been shown to induce biochemical effects in the circulation (Perumpanani et al. 1998).

The physical properties of the ECM allows it to function as an important store of growth factors and also regulates growth factor activity (Araya and Nishimura 2010), as these cytokines are often stored in latent form within the ECM (Raghunath et al. 1998). Fibrotic growth factors, like TGFβ1, in turn can alter the stiffness of the matrix by increasing production of ECM molecules like fibronectin and collagen.

The importance of a tightly regulated matrix stiffness lies in the fact that cells respond to mechanical signals as well as chemical ones. The expression of the contractile protein α-smooth muscle actin (α-SMA) by pulmonary fibroblasts *in vitro* has been shown to be dependent on the stiffness of the surface they are seeded on while substrate stiffness can also alter the sensitivity of cells to cytokines (Chia et al. 2012).

Therefore, understanding the role of the ECM as a controller of tissue homeostasis is key in understanding fibrotic disease progression.

### 1.3.3 Tissue homeostasis and ECM turnover

The complexities of cell turnover are outside the scope of this thesis, but it is important to point out that as cells are being turned over, so too is the underlying structure that holds them all together (Pardo and Selman 2001).

In the normal organ, there is continuous ECM deposition and degradation, known as tissue homeostasis. Tissue homeostasis is necessary to replace any dead or damaged cells in the body. The rate of cell turnover differs in each organ. For example, lung epithelium takes six months to regenerate whereas the lining of the gut is thought to turnover every five days (Blanpain et al. 2007) .

Dysregulation of the MMPs and TIMPs is a shared mechanism of metastasis in multiple cancers (Ricciardelli and Rodgers 2006) and is also seen in pulmonary fibrosis (Corbel et al. 2002).

### 1.3.4 Cells that produce ECM

While all cells produce varying amounts of ECM, there are two categories of cells that are responsible for the majority of the structure known collectively as the ECM in the lung.

#### 1.3.4.1 Lung mesenchymal cells

The type of mesenchymal cells that are the major contributor to the ECM are fibroblasts. Fibroblasts are difficult to define as they lack a unique and universal cell-surface marker, despite being present in most tissues (Burstein et al. 2008).

Fibroblasts themselves are not a homogeneous population of cells. Fibroblast sub-types are differentiated in the tissue they are found in. In the lung, the heterogeneity of fibroblasts from different areas of the lung is specifically dysregulated depending on the type of lung disease present (Kotaru et al. 2006). In IPF, fibroblasts from areas undergoing rapid fibrotic change show increased responsiveness to inflammatory cytokines compared to fibroblasts from areas of slower fibrotic change (Habiel and Hogaboam 2014). Fibroblasts are also potent sources of mediators that recruit other cells to the site of injury, such as eotaxin which attracts eosinophils (Kotaru et al. 2006).

Similarly, circulating fibroblast progenitor cells, known as fibrocytes, are thought to home in on areas requiring sudden ECM deposition (Andersson-Sjoland et al. 2008). The contribution of the fibrocyte has been identified in renal fibrosis, and airway remodelling in asthma and there is a positive correlation between the number of lung fibrocytes and the abundance of fibroblastic foci in patients with IPF (Hardie et al. 2009), a potential marker of disease severity (Flaherty et al. 2003).

#### 1.3.4.2 Lung epithelial cells

The main function of epithelial cells is maintenance of barrier function. The epithelial lining of the airway system is divided into 3 anatomically distinct regions, the trachea/bronchi, the bronchioles, and the alveoli. All three areas contain epithelial cells that appear typically cobblestone-shaped in monolayer cell culture (Rackley and Stripp 2012). Major cell types of the airways include ciliated, columnar, undifferentiated, secretory and basal cells (Hackett et al. 2008).

In the alveolar region, type I and type II pneumocytes line branches $2^{23}$ and greater. 90% of the alveolar surface is covered in type I cells to provide a surface suitable for

gas exchange. In concert, type II cells are found closer in proximity to mesenchymal cells and serve as surfactant producers and type I progenitors (Selman and Pardo 2006).

### 1.3.5 Important ECM proteins

Due to the incredible protein diversity that is exhibited by the ECM, we have chosen to investigate a few ECM proteins of note in this study that have previously been identified to play roles in fibrogenesis. Collagen and fibronectin make up the majority of the ECM as they provide the main structural components. Periostin and tenascin-C play important roles in the orientation of collagen and fibronectin respectively (Kii et al. 2010).

#### 1.3.5.1 Fibronectin

Fibronectin is one of the most highly conserved ECM proteins in the animal kingdom and its assembly into the ECM is a cell-mediated, integrin-dependent process (Mao and Schwarzbauer 2005). During this assembly, fibronectin is required to undergo many conformational changes that allow for the exposure of the necessary binding sites for other proteins needed to assist in the process (Sottile and Hocking 2002).

Aside from directly influencing cell behaviour such as proliferation, attachment, migration and survival, the formation of a scaffold of fibronectin guides the deposition of latent TGFβ binding proteins that regulate the storage of TGFβ and therefore influence its activity (Dallas et al. 2005). Briefly, soluble fibronectin dimers are dispersed diffusely over the cell surface. Next these dimers are arranged into short fibrils and finally into a fibrillar network (Mao and Schwarzbauer 2005).

Fibronectin has been shown to be critical in development as mice lacking in the fibronectin gene die in early embryogenesis due to defects in cell migration (Muro et al. 2008). Furthermore, fibronectin is an essential part of the wound provisional matrix and is a major blood protein (Midwood et al. 2006). A functioning fibronectin scaffold is necessary for the deposition of collagen I (Sottile and Hocking 2002), one of the other main components of the ECM.

#### 1.3.5.2 Collagen

Collagen is the most abundant protein in the ECM, accounting for about 30% of the total weight of the ECM. In addition, there are dozens of collagens that differ in function and ECM abundance (Prockop and Kivirikko 1995). It is type I collagen that is induced upon treatment with bleomycin (Reiser and Last 1983), a drug typically prescribed for cancer chemotherapy which has been shown to cause pulmonary fibrosis (Mouratis and Aidinis 2011). Collagen fibrils are most responsible for the strength of the tissue and define an organ's shape. The generation of these fibrils is an important step to understand in the context of both development and disease and this process is still not fully elucidated. The induction of collagen by TGFβ1 is one of the most well-studied effects of pro-fibrotic cytokines (Kenyon et al. 2003). The exact mechanisms driving collagen deposition are outside the scope of this thesis, but collagen maturation is important to mention as all scar formation is made of immature collagen (Widgerow 2011).

Pro-collagen fragments are translocated to the lumen of the endoplasmic reticulum (ER) where it co-localises with other molecular chaperones and enzymes necessary for correct folding and polymerization (Lamande and Bateman 1999), similar to fibronectin fibrillogenesis. Fibrillogenesis of the major structural components, both fibronectin and collagen, is a tightly organized process involving other ECM proteins

that act as molecular bridges. Previously identified molecular bridges of collagen include decorin (Culav et al. 1999) and periostin (Norris et al. 2007).

#### 1.3.5.3 Periostin

Periostin is a secreted ECM protein that is associated with collagen-rich fibrous connective tissues and directly controls proper collagen I fibre formation (Norris et al. 2007). Periostin was first described as a TGFβ-induced protein that was expressed following inflammation during tooth and bone remodelling (Kudo 2011). In addition to TGFβ, periostin is also upregulated by inflammatory cytokines such as interleukin (IL)-13 and contributes to the airway remodelling seen in asthma (Takayama et al. 2006).

During remodelling, periostin enhances the activity of lysyl oxidase (LOX), the enzyme responsible for collagen cross-linking, through interactions with both fibronectin and bone morphogenic protein (BMP) -1 (Maruhashi et al. 2010). Furthermore, periostin also acts in concert with tenascin-C, possessing adjacent domains to those on tenascin-C that bind to fibronectin and type I collagen. Deposition of tenascin-C into the ECM was shown to be dependent on the presence of periostin (Kii et al. 2010).

#### 1.3.5.4 Tenascin C

Tenascin-C is a member of the tenascin family which includes other tenascins such as tenascin-X and tenascin-H (Udalova et al. 2011). It is only expressed during periods of active tissue repair and is downregulated once repair is complete. As a hexametric molecule with multiple binding sites, tenascin-C exhibits the ability to bind to and regulate a variety of cell-cell and cell-matrix interactions (Midwood et al. 2011). In

this manner, tenascin-C can be thought of as a counter to the adhesive actions of fibronectin.

Similar to fibronectin and other ECM protein binding partners, tenascin-C can also be induced by TGFβ1 (Hau et al. 2006). In addition, tenascin-C participates in the fibrinolytic system through its ability to inhibit plasminogen, the precursor to plasmin, the enzyme that degrades fibrin (Brellier et al. 2011). The fibrinolytic system plays an important role during normal wound healing and is vital in dampening fibrosis (Swaisgood et al. 2000).

## 1.4 Fibrosis

### 1.4.1 Regeneration vs Repair

Damage as a consequence of mere existence is a problem shared by all living organisms. Tissue homeostasis is maintained through tightly regulated cell turnover during which older cells are replaced by newer ones. During this process of regeneration, normal tissue morphology and function are preserved. In a review of tissue homeostasis, authors Pellettieri and Alvarado state *"it has been estimated that each of us eradicates and, in parallel, generates a mass of cells equal to almost our entire body weight each year" (Pellettieri and Sanchez Alvarado 2007).*

In contrast to this, reconstitution of an adult tissue that does not exactly replicate the original state is known as repair (Martin and Parkhurst 2004). Fibrosis can be thought of as pathological wound healing (Diegelmann and Evans 2004). The process of fibrosis is part of the natural wound healing process that is necessary for development and growth as well as repair. However, somewhere along this process, the regulation of ECM deposition goes awry. The end result is excessive ECM deposition that

changes the architecture of the organ that is affected. Similarities between fibrogenic lung diseases have been repeatedly reviewed throughout the literature (Araya and Nishimura 2010; Murray 2012).

### 1.4.2 The stages of wound healing

During wound repair, one of two things can occur. Either the wound can be mended with fibrotic scar tissue, or it can be replaced with normal, and ordinarily functioning, tissue. Some species are known to have great systems to keep that repair process in check. The scar-free repair process following tail loss in the gecko is a perfect example of this (Delorme et al. 2012)(Figure 1.4) .

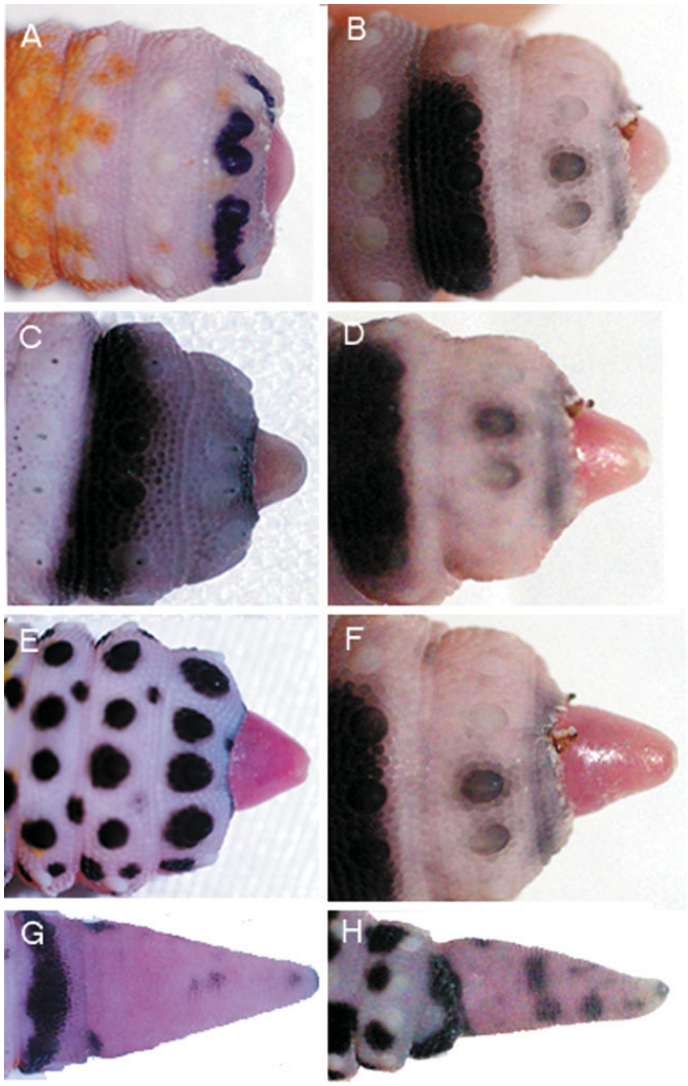



**Figure 1.4 Images of scar-free wound healing and regeneration following tail loss in the leopard gecko.**
The gross morphology of the tail following (A, C, E, G) autonomy, the self-detaching of the tail, or (B, D, F, G) amputation is indistinguishable from one another.
Reproduced with permission from (Delorme et al. 2012)

The gecko, and many other urodeles (tailed amphibians), can spontaneously regenerate a lost tail, without any scarring occurring during the repair process (Delorme et al. 2012). While the regenerative capabilities of these animals are outside the scope of this thesis, it would be also be interesting to study the processes active in those animals in contrast to the formation of scar tissue that is a possible, and unwanted, conclusion of wound repair (Yates et al. 2011).

In humans, wound healing is a complicated process involving the initiation and resolution of a number of different stages. Each stage involves many different cell types, proteins and signalling pathways. Haemostasis is the first stage that involves the cooperation of the coagulation system as well as the fibrinolytic system. During haemostasis the formation of the fibronectin provisional matrix occurs and its development is controlled by members of the coagulation cascade (Mosher 1995). The ECM molecule fibrin is a fibrous protein formed from fibrinogen and is polymerized to form the mesh work that plugs the wound (Reinke and Sorg 2012). After haemostasis at the wound site is complete, the stages of wound healing are classically divided into three parts, (1) inflammation, (2) proliferation, (3) remodelling (Figure 1.5).

During the inflammation stage, neutrophils are the cell type most abundant and they move from the circulation into the wound microenvironment as guided by chemokines and other chemotactic agents, like ECM proteins. Neutrophilic phagocytosis removes any foreign material, bacteria, dead cells and bits of damaged ECM. Also present in the wound microenviroment are mast cells, monocytes and

macrophages. These cells provide the chemical mediators to activate the structural cells for the next stage of wound healing (Diegelmann and Evans 2004).

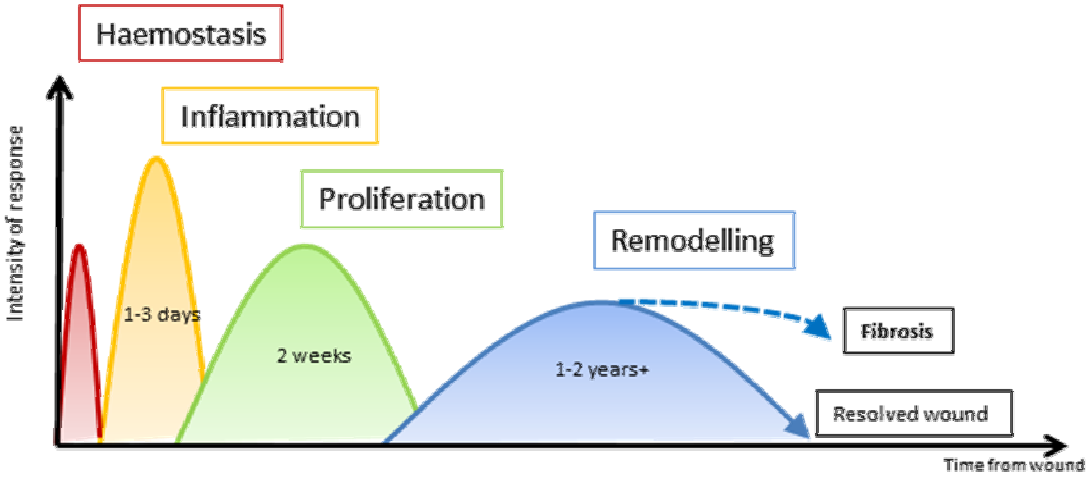



**Figure 1.5 The stages of wound healing**
Wound healing is a series of events that commence and conclude in series. The failure to resolve the final stage, remodelling, results in fibrosis.
Adapted from www.worldwidewounds.com accessed September 2013.

Following inflammation, proliferation is required to replace the cells that have been lost and to start knitting together the edges of the wound. TGFβ1 is a critical cytokine in this process and will be covered in greater detail later in this chapter. TGFβ1 increases the deposition of many ECM molecules, in particular, but by no means limited to, collagen (Sidhu et al. 2010). At the same time, TGFβ1 can inhibit proteases involved in ECM turnover and increase inhibitors of those matrix proteases to further encourage ECM deposition (Sidhu et al. 2010).

Epithelisation is driven by cytokines produced by platelets and macrophages (Reinke and Sorg 2012). New blood vessels begin to form at the wound site, stimulated by growth factors produced by vascular endothelial cells. Fibroblasts produce the

increased quantities of ECM proteins needed to create the supramolecular structures that stabilize the newly formed tissue for the next phase of the process.

Resolution of wound healing involves the remodelling of the tissue architecture. Fibroblasts are the connective tissue cells that are responsible for the ECM deposition needed to repair the injury. Increased ECM deposition is necessary to replace structures that were lost. Before proliferation decreases, the edges of the wound need to be brought together in a process that is regulated through the fibroblasts' contractile properties (Tomasek et al. 2002).

The idea is that by the end of this process (1-2 years or longer) the acute inflammatory and proliferative environment at the wound site is no longer needed. As the wound heals, the number of fibroblasts and macrophages should decrease by apoptosis and the growth of blood capillaries stops (Bonner 2010). If there is too little deposited ECM at the wound site, the repair will be weak and may split apart. However, if too much ECM is deposited then the normal architecture of the site is compromised and anatomical function is lost. This is called fibrosis.

### 1.4.3 The ECM in fibrosis

Fibrosis in humans is thought to follow a similar pattern no matter which is the organ/tissue that is affected (Thannickal et al. 2004). When the fibrosis is in the lung, it is termed pulmonary fibrosis. This term is often confused with the named types of ILD, such as IPF, and should be thought of as a consequence of many different diseases, not a disease in and of itself.

A common reoccurring theme of fibrosis, no matter the organ affected, is the disruption of one or more of the regulatory processes that control the ECM. Because

there is not a particular protein that is only found in areas of active fibrosis and ECM production and turnover of the ECM is a normal part of homeostasis, understanding the underlying mechanisms and key ECM components contributing to fibrosis is essential. However, little attention has been paid to the exact composition of the ECM as it changes throughout development and during stages of disease (Royce et al. 2009; Booth et al. 2012).

### 1.4.4 Dysregulation of ECM in pulmonary fibrosis

Change to ECM protein organization is an essential step in the development and restoration of organ function and similarities between these two processes have been recognized in the context of fibrotic disease (Martin and Parkhurst 2004; Redd et al. 2004; Hardie et al. 2009; Imanaka-Yoshida 2012). In pulmonary fibrosis, dysregulation of particular ECM proteins can be linked to every stage of wound healing.

As one of the initial stages following damage, the importance of the fibrinolytic system has been implicated in pulmonary fibrosis as mice that do not express either urokinase or plasminogen exhibited accelerated fibrosis in response to bleomycin treatment (Swaisgood et al. 2000). This pro-fibrinolytic system is diminished in the alveolar microenvironment of lung diseases (Sisson and Simon 2007) while tenascin-C levels are increased in the serum of patients with collagen diseases (Inoue et al. 2013). Plasmin is secreted from the liver as plasminogen, which is inactive in the circulation. Tenascin-C was shown to inhibit the conversion of plasminogen to plasmin by downregulating tissue plasminogen activator (Brellier et al. 2011). Plasmin is responsible for the degradation of the fibrin that is present in the provisional wound matrix.

Although IPF is no longer thought to be driven by inflammation, abnormal wound repair in the alveolar microenvironment is thought to propagate pulmonary fibrosis from the epithelium to the interstitium (Selman and Pardo 2006). During the inflammatory phase, ECM proteins like periostin (Takayama et al. 2006) are released from damaged epithelial cells and contribute to subepithelial fibrosis found in the context of asthma.

Release of pro-fibrotic mediators like TGFβ1 and platelet-derived growth factor (PDGF) by both immune cells, like mast cells (Thomas et al. 2010) and macrophages (Lacronique et al. 1984), as well as by epithelial cells becomes excessive in fibrosis (Leask and Abraham '2004). Cytokine production also induces the proliferation and activation of fibroblasts that drive fibrosis (Kramann et al. 2013) and results in ECMs with an altered protein profile (Booth et al. 2012).

This altered ECM profile, the result of chronic injury, slowly disrupts the resolution of the wound (Yates et al. 2011). Furthermore, ECM deposition becomes unrestrained as the mechanisms regulating survival of mesenchymal cells are decreased (Bonner 2010). The effect of the ECM on cell survival is well documented in the context of cancers (DeClerck 2010) however the contribution of small ECM proteins has not been fully examined.

Composition of the ECM is important because the particular make-up of the ECM defines both its biomechanical and biochemical properties (Antunes et al. 2009). In the context of fibrotic lung disease, it has long been established that the lung becomes stiffer as the disease progresses (Gibson and Pride 1976). The result of increased stiffness may be important in the pathogenesis of fibrosis as fibroblasts from patients with scleroderma express increased levels of contractile ECM proteins and lower

levels of elastic fibre content (Reich et al. 2009). However, there have been few studies relating the changes to matrix stiffness with alterations in the ECM profile in patients with IPF.

### 1.4.5 Matrix Stiffness

Physical effects of the ECM are of particular importance in the lung. The ECM is responsible for the compressive and tensile strength of the tissue or organ. Increases in ECM stiffness relate to the progression of fibrosis of the lung and increase the work of breathing (Faffe and Zin 2009). The stiffening of lung tissue has previously been regarded as the passive end-point of the fibrotic process. In fact, more modern concepts describe how fibroblasts and other cells respond to increased matrix stiffness by selectively increasing expression of contractile proteins and decreasing production of fibrosis-inhibiting cytokines (Liu et al. 2010; Mih et al. 2012).

### 1.4.6 Atomic Force Microscopy

Matrix stiffness can be measured using a technique that has long been employed in research on inorganic surfaces. In 1988, atomic force microscopy (AFM) on an organic material showed that the forces used for imaging had no effect on a monolayer of polymer strands and could potentially be used to study biological systems (Marti et al. 1988).

AFM can measure the amount of deflection that a cantilever experiences as it travels over the surface of the fibroblast (topography). In addition, it can also measure the amount of resistance to deflection, also known as stiffness, of each point on that surface. We can then overlay the two measurements and obtain both the surface morphology and underlying stiffness of fibroblasts grown in a monolayer. In addition

to the ability to measure basal differences between normal and fibrotic fibroblasts (Reich et al. 2009), it is also possible to measure the changes in matrix stiffness as a result of stimulation with growth factors such as TGFβ1(Chia et al. 2012).

## 1.5 Transforming growth factor-β1

### 1.5.1 Background

TGFβ1 is a multifunctional cytokine that is one of the most effective at promoting the fibrotic microenvironment. Upon activation of TGFβ1, a complicated series of signalling events is set off, involving multiple pathways, within and external to the cell. Because multiple cellular pathways are affected, TGFβ1 is able to influence many different phenotypes and biological processes including cancer and wound healing.

In particular, TGFβ1 promotes the fibrotic phenotype by increasing the expression of many ECM proteins including but not limited to, collagen, fibronectin, tenascin C and periostin. Furthermore, TGFβ1 decreases the production of MMPs and increases TIMPs, resulting in greater ECM conservation (Nagase et al. 2006). Due to the accumulation of latent TGFβ1, within the ECM, a greater amount of ECM results in a greater store of potentially active TGFβ1 (Lepparanta et al. 2012).

Because of the pro-fibrotic environment it generated, it was quickly demonstrated that TGFβ1 plays a critical role in the development of fibrotic lung disease (Hoyt and Lazo 1988). Dysregulation of fibroblasts and epithelial cells play a central role in the mechanisms that drive pulmonary fibrosis. Firstly, TGFβ1 can delay epithelial cell proliferation and migration while promoting apoptosis (Lee et al. 2004). Secondly, TGFβ1 is a major growth factor that regulates the conversion of a quiescent fibroblast

to the contractile alpha-smooth muscle actin (αSMA) expressing myofibroblast (Horowitz and Thannickal 2006). Finally, TGFβ1 signals the recruitment of inflammatory cells to the site of the insult (Diegelmann and Evans 2004). All these cells also produce cytokines that mediate TGFβ1 activity (Bonner 2010).

### 1.5.2 Cytokines, the ECM and TGFβ1 regulation

TGFβ1 is secreted in an inactive complexed form and targeted to specific places in the ECM by the latent TGFβ1 binding protein (LTBP) (Hyytiainen et al. 2004) (Figure 1.6).

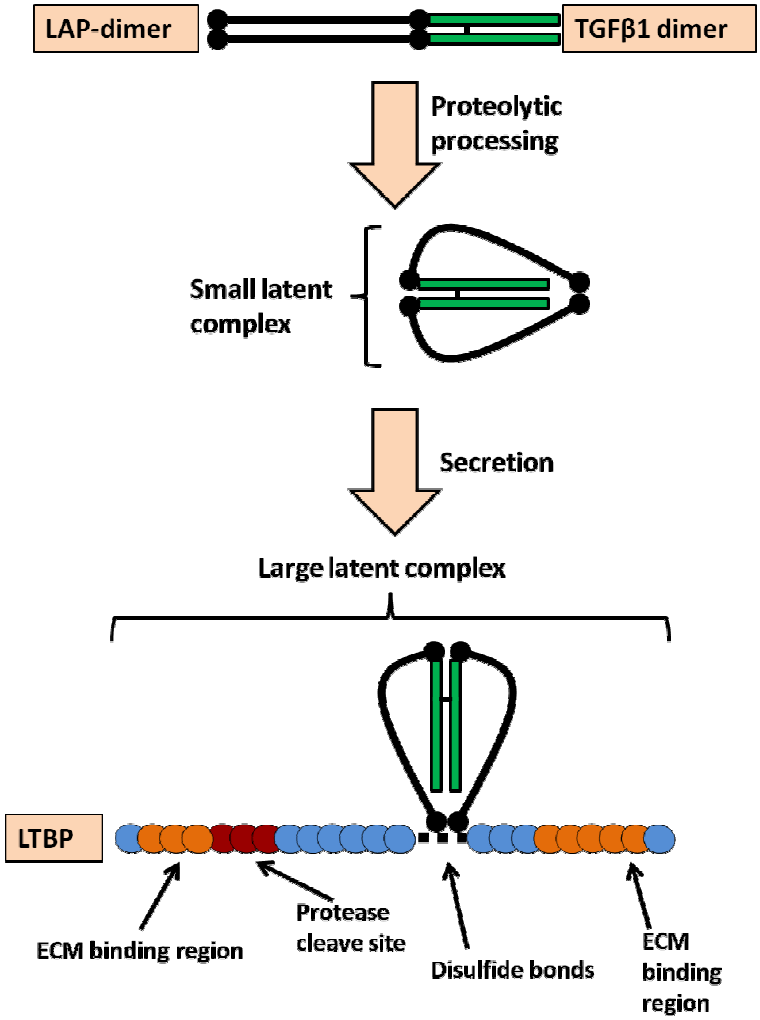



**Figure 1.6 The formation of the small and large latent forms of transforming growth factor-beta 1 (TGFβ1).**
TGFβ1 is secreted and stored in the lung extracellular matrix (ECM) in an inactive form through the direction of the latent TGFβ1 binding protein (LTBP). Activation of TGFβ1 requires removal of the latency binding peptide (LAP).
Adapted from (Hyytiainen et al. 2004)

The large latent complex is bound to the ECM through ECM binding regions at the amino and carboxyl terminals of the LTBP. The small latent complex is released from the ECM through protease cleavage sites but activation of TGFβ1 requires the additional removal of the non-covalently bound latency binding peptide (LAP) from the TGFβ1 dimer (Dubois et al. 1995).

The activity of TGFβ1 is both pro-inflammatory and anti-inflammatory and is mediated through several mechanisms. Due to the exceedingly complex array of pathways affected by TGFβ1, only those mechanisms known to be pertinent to the role of TGFβ1 in the lung will be mentioned. It is not surprising that in the human lung, the means by which regulation and storage of TGFβ1 is accomplished have not been fully elucidated (Lepparanta et al. 2012).

TGFβ1 signals by initially binding to the TGFβ type II receptor which then recruits TGFβ type I receptor on the cell membrane. The receptor complex activates the Smad2/3/4 complex, causing it to be transported to the nucleus and combine with transcriptional coactivators CREB-binding protein (CBP) and p300. In addition, the TGFβ1 receptor complex can also activate Smad-independent pathways such as through c-Jun N-terminal kinase (JNK).

TGFβ induces many ECM proteins such as perlecan (Ichimaru et al. 2012) and collagen (Kenyon et al. 2003), as well as growth factors such as connective tissue growth factor (CTGF) (Kono et al. 2011), vascular endothelial growth factor (VEGF) (Lee 2012) and fibroblast growth factors (FGF) (Srisuma et al. 2010). TGFβ1 can also increase ECM proteases such as matrix metalloproteinases MMP2 and MMP9 (Dallas et al. 2002). All of these factors are likely to be important in the development of fibrosis.

### 1.5.3 TGFβ1, EMT and pulmonary fibrosis

TGFβ1 is overexpressed in many pathological conditions that have a fibrotic component such as pulmonary fibrosis (Lepparanta et al. 2012), kidney disease (Lopez-Hernandez and Lopez-Novoa 2012), Crohn's disease and cancer (Prud'homme 2007). LTBP-1 expression was found to be increased in the lungs of patients with IPF (Lepparanta et al. 2012). In addition, increased active TGFβ1 is found in the bronchoalveolar lavage (BAL) of patients with IPF compared to normal controls (Khalil et al. 2001).

Pulmonary fibrosis is the consequence of a range of acute and chronic lung injuries that eventually culminate in the destruction of the normal lung architecture and death (Araya and Nishimura 2010). It has been shown through tissue specimens of patients with pulmonary fibrosis that it is an abnormal wound response driving excessive ECM deposition (Thannickal et al. 2004; Araya and Nishimura 2010). As a pleiotropic cytokine, TGFβ1 can tip the cellular phenotype of epithelial cells towards a pro-fibrotic phenotype (Doerner and Zuraw 2009) making it a critical contributor to the pulmonary fibrosis in interstitial lung disease (Strieter 2008).

Previous work in our laboratory has shown that TGFβ1 increases the production of the ECM protein fibulin-1 and which plays a role in the pathophysiology of airway fibrosis in asthma (Lau et al. 2010). Fibulin-1 may play an important role in the pathophysiology of diseases such as IPF, which are predominately characterized by diffuse pulmonary fibrosis.

## 1.6 Fibulin-1

### 1.6.1 Background

Fibulin-1 is an ECM glycoprotein that was first discovered as an unknown binding partner of fibronectin (Argraves et al. 1989). Further study into the characterization of fibulin-1 showed that it was a calcium-binding protein with a repeated structure and was expressed in cultured gingival fibroblasts (Tanaka et al. 1994). One of the first described roles of fibulin-1 was its association with elastic fibres (Roark et al. 1995).

Fibulin-1 was found in the unstructured core of elastic fibres but not in the fibrillin-containing, elastin-associated microfibrils, suggesting that fibulin-1 was a structural protein with a role in the elastic properties of connective tissue fibres (Roark et al. 1995). Secreted fibulin-1 becomes incorporated into a fibrillar ECM when added exogenously to cultured fibroblast monolayers (Argraves et al. 1990), and recent studies have shown that fibulin-1 ECM production is in part driven by TGFβ1 (Chen et al. 2013). In the blood, fibulin-1 is the predominant fibulin, with a reported plasma concentration range of 30-40µg/mL (Tran et al. 1995). In addition, fibulin-1 mRNA is found in most tissues and in some cultured cells (Roark et al. 1995).

Previous studies have identified the role of fibulin-1 in asthma (Lau et al. 2010). In asthma, the generation of airway fibrosis is among a variety of structural changes known collectively as airway remodelling (Elias et al. 1999). The degree of airway remodelling, similar to the degree of parenchymal remodelling, has been linked to increased disease severity (Shifren et al. 2012). The extent of fibrosis, or the degree of parenchymal consolidation, has been used as a measure of disease severity in IPF (Wells et al. 2003).

Similarities between fibrotic lung diseases outline the potential for common mechanisms of fibrogenesis in the lung (Araya and Nishimura 2010). Fibulin-1 may be involved in the general mechanism that is essential for normal wound healing and dysregulation of fibulin-1 may have a role in the pathogenic changes that occur during lung fibrosis.

### 1.6.2 The fibulin family

The fibulin family of ECM proteins currently sits at eight identified members which all share a similar elongated structure with overlapping binding sites for several basement-membrane proteins. Fibulin-1 was the first to be identified (Argraves et al. 1989) and was followed by discoveries of fibulin-2 (Pan et al. 1993), fibulin-3 and fibulin-4 (Giltay et al. 1999), and fibulin-5 (Kowal et al. 1999), also known as DANCE (Nakamura et al. 2002). Fibulin-6 (Katoh and Katoh 2004), also known as HMCN1, and fibulin-7 are recently added members of the fibulin family. Fibulin-7 is also a binding partner of fibulin-1 (de Vega et al. 2007).

Orthologs of fibulins are seen in all animals (Segade 2010) with seven of the fibulins found in mammals (Vogel et al. 2006). Fibulin-8 is the last of the fibulins that have been described and is not found in any mammals, except the platypus (Segade 2010) (Figure 1.7).

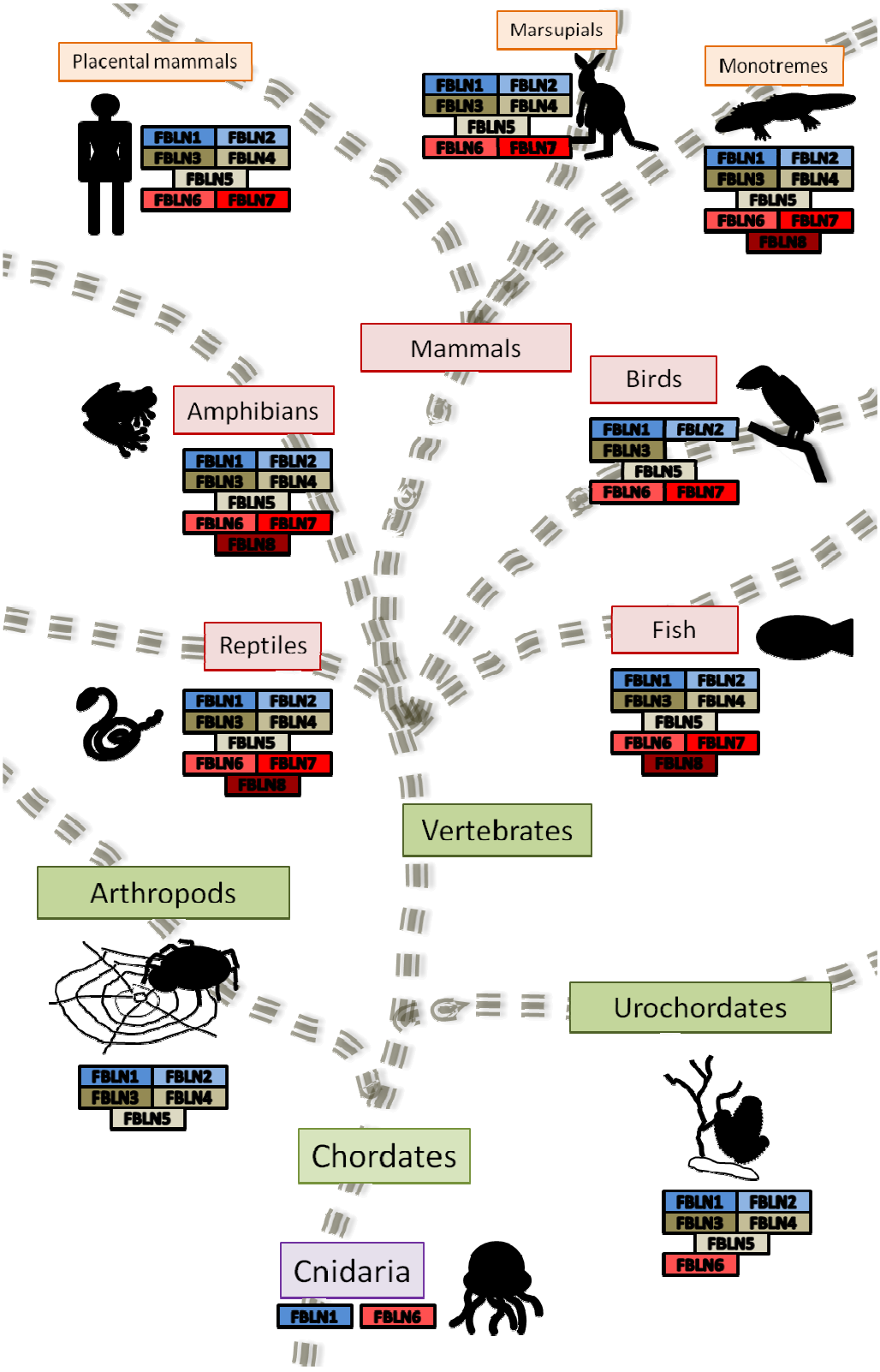



**Figure 1.7 The molecular evolution of the fibulins (FBLN).**
The FBLN genes are highly conserved throughout the animal kingdom. There are eight FBLNs known to date.

### 1.6.3 The structure of fibulin-1

Fibulin-1 and its family members share a similar repeated domain structure. Named after the latin fibula, meaning clasp or buckle, members of the fibulin family are widespread components of the ECM whose protein structures are all arranged in a distinct form. These repetitive domains are necessary for the protein interactions involved in ECM assembly (Hynes 2009).

Their amino acid sequences are grouped into modules named domain I, II and III (Figure 1.8). Domain I is the amino-terminal sequence that varies among fibulin members. Domain II contains a variable number of repeated epidermal growth factor like modules. The specific part of the sequence is domain III, also known as the fibulin C-terminal globular (FC) domain which is shared between 7 of the fibulin members (Fibulin-1 to 7) (de Vega et al. 2009).

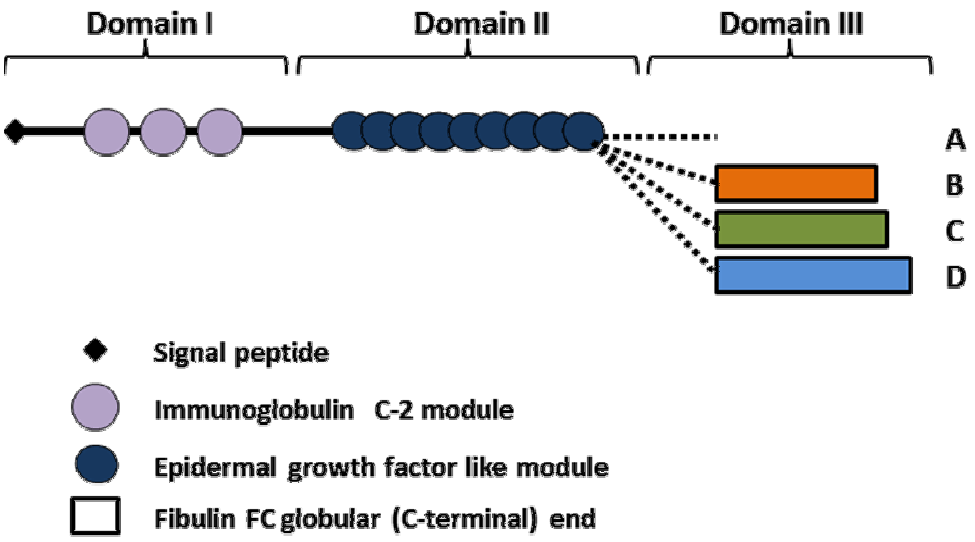



**Figure 1.8 The structure of fibulin-1**

This repetitive domain structure is a reflection of the ancient origins of the fibulin family. The highly conserved structure can be seen in the multiple orthologs that are shared by all vertebrates (Vogel et al. 2006) and in fact, the origin of the fibulin gene family has been traced to the base of the metazoans, or animal kingdom (Segade 2010). The simplest of the metazoans are nematodes (flat worms) and mammals share both fibulin-1 (Hesselson et al. 2004; Kubota et al. 2004) and fibulin-6 (Vogel and Hedgecock 2001) with them. As a result of this interspecies homology, a lot of the study into the function of fibulin-1 has been carried out in "simpler" model systems.

Evolutionarily speaking, the fibulin family can be described as basement membrane proteins (Argraves et al. 2003), and some, like fibulin-1, have involvement in elastic fibre assembly (Roark et al. 1995). Fibulin-1 is 90-100kDa and gene splicing results in 4 isoforms of varying C-terminal domains (A-D) (Figure 1.8).

### 1.6.4 Fibulin-1 has 4 isoforms

The functions of the four fibulin-1 isoforms have not been fully elucidated. The natural expression of fibulin-1A and fibulin-1B is in the human placenta, and at low abundance. Fibulin-1A and B have also been shown to be expressed in ovarian tissues and cancer cell lines at trace amounts (Moll et al. 2002). Alternative splicing of the gene gives rise to proteins of varying lengths (Table 1.1**).** For proper processing through the golgi apparatus, each fibulin-1 isoform undergoes post-translational modification where an identical 29-amino acid signal peptide is added to Domain I.

**Table 1.1 The size and sequence of the fibulin-1 isoforms in humans**

| Isoform | # Amino acid residues | GenBank Accession number |
|---|---|---|
| A | 537 | NM_006487 |
| B | 572 | NM_006485 |
| C | 654 | NM_001996 |
| D | 656 | NM_006486 |

All four isoforms of fibulin-1 share the first 537 amino acid residues with fibulin-1A completely missing the C-terminal domain III (Tran et al. 1997). Only the two longer fibulin-1 isoforms, C and D, contain the fibulin-type carboxy-terminal module (Fujimoto et al. 2005). The predominant fibulin-1 forms in humans are isoforms C and D (Roark et al. 1995) and these are proteins of similar molecular weight (100kDa) but whose functions are thought to be distinct (Moll et al. 2002). However, functional

redundancy between fibulin-1C and D and fibulin-6 has been shown, albeit in zebrafish (Feitosa et al. 2012).

In *Caenorhabditis elegans (C. elegans),* the flatworm model organism which was the first to get its genome sequenced (Sulston and Brenner 1974), the function of fibulin-1C was shown to be involved in the control of developmental growth through the epidermal growth factor (EGF)-like repeats (Hesselson and Kimble 2006). *C. elegans* fibulin-1C had specific roles in the development of the pharynx, intestine, gonad and muscle. Conversely, fibulin-1D was shown to assemble in the flexible polymers that connected the pharynx and basement membranes of the body wall (Muriel et al. 2005). However, in humans, the EGF domain of fibulin-1 is the same for all fibulin-1 isoforms (Argraves et al. 1990). The nucleotide length difference between fibulin-1C and fibulin-1D is a mere 0.6kb but they share only approximately 28% identity with each other's C-terminal domain (Tran et al. 1997). The isoform specific functions of human fibulin-1 have not been fully examined.

Dysregulation of the isoform balance has been reported in some cancers (Moll et al. 2002) but not others (Wlazlinski et al. 2007). Fibulin-1C has been linked to tumour progression (Moll et al. 2002) whilst haplo-insufficiency of the fibulin-1D gene results in limb malformations seen in synpolydactyly (Debeer et al. 2002). In mice, isoforms C and D were found to differ in their affinity for nidogen, another important ECM protein (Sasaki et al. 1995). Furthermore, the assembly of fibulin-1C into the ECM was shown to be dependent on the basement membrane protein laminin in *C. elegans*, but the inclusion of fibulin-1D was linked to perlecan, another component of the basement membrane (Muriel et al. 2006). This indicates that it is likely that there

are site-specific functions to fibulin-1 isoforms that have been conserved over a long period of time.

However, investigations of fibulin-1 often do not specify which isoform is being studied. Despite this limitation in the literature, the common modules of fibulin-1 confer properties of interest as the vast majority (~80%) of each isoform is of identical sequence.

### 1.6.5 Fibulin-1 protein-protein interactions

The ability of fibulin-1 to participate in supramolecular structures stems from the presence of overlapping binding sites with several other basement-membrane proteins such as tropoelastin, fibrillin, and fibronectin (Timpl et al. 2003) in addition to the aforementioned nidogen (Sasaki et al. 1995), laminin and perlecan (Muriel et al. 2006). Fibulin-1 is also known to bind to aggrecan and versican (Aspberg et al. 1999) as well as nidogen (Sasaki et al. 1995). Nidogen is part of the CCN family of growth factors of which connective-tissue growth factor (CTGF) is also a member (Perbal 2001).

Fibulin-1 can also enhance the properties of other ECM proteins such as the metalloproteinase A Distintegrin And Metalloprotease with Thrombospondin repeats (ADAMTS)-1 (Lee et al. 2005). Fibulin-1C was shown to bind to the C-terminal domain of NOVH protein, which is a negative regulator of cell growth and member of the CCN family (Perbal et al. 1999). One way that fibulin-1 exerts influence is by modulating the bioactive effects of fibronectin, one of the main ECM proteins.

### 1.6.6 Fibulin-1 and fibronectin

As the molecule responsible for the discovery of fibulin-1, fibronectin-fibulin-1 interactions may be a confounding factor in this study. As previously mentioned in this Chapter, fibronectin is an essential ECM player that influences proliferation, attachment, migration and growth factor storage. Fibronectin is also a highly expressed protein found in the blood, having been reported at concentrations of 300-350µg/mL. Serum fibronectin levels are also elevated in, and were able to discriminate between those with liver fibrosis and those with non-fibrotic livers (Attallah et al. 2013). As fibronectin is required for collagen deposition, it is possible that increased fibronectin levels contribute to increased pulmonary fibrosis.

In ILD, fibronectin levels are elevated in the BALF but not in the plasma of patients with IPF, sarcoidosis and "other" ILDs compared to healthy controls and patients with non-interstitial lung diseases (Rennard and Crystal 1982). Surprisingly, the most recent study on the levels of fibronectin in patients with ILD was published in 1994 (Zhao et al. 1994). However, plasma levels of fibronectin were found to be elevated in asthmatics compared to healthy controls (Ohke et al. 2001) and studies undertaken by a previous PhD student in this laboratory have shown that fibulin-1 is increased in the serum and BALF of asthmatics (Lau et al. 2010). It is possible that with advances in methodologiess with which to distinguish the different forms of fibronectin, that an increase in the levels one form of fibronectin in the serum of patients with IPF may be identified. As fibronectin and fibulin-1 bind together, this could therefore influence the levels of fibulin-1 in patients with IPF.

### 1.6.7 Fibulin-1 and collagen

Collagen is the main component in fibrosis and but its relationship to fibulin-1 has not been studied in great detail. Collagen fibrillogenesis is a complex cell-mediated process that involves helper molecules like periostin (Kudo 2011) and possibly fibulin-1. Decades old analysis on fibulin-1 binding activity demonstrated that fibulin-1 binds to collagen IV but not to collagen I, II, III, V and VI (Sasaki et al. 1995). During gonadogenesis in the *C. elegans,* the orthologs to mammalian fibulin-1 and ADAMTS act antagonistically against each other to control tissue architecture involving collagen IV (Kubota et al. 2012). In a model of IPF, alveolar epithelial cells produced biologically active TGFβ1 and increased deposition of collagens I, III, V and in particular IV (Xu et al. 2003), highlighting another potential mechanism by which fibulin-1 may play a role in pulmonary fibrosis.

Fragments of collagens (type I, III, V, and VI) have been reported as increased in the serum of patients with IPF, stressing the potential of ECM molecules as biomarkers (Leeming et al. 2012).

### 1.6.8 Fibulin-1 and periostin

Both fibulin-1 and periostin function as regulators of collagen fibre formation (Norris et al. 2007) and periostin is the only ECM molecule that has been identified as a biomarker of disease progression in IPF (Naik et al. 2012). Like fibulin-1, periostin acts as a bridging molecule which alludes to an overarching mechanism by which dysregulated large fibre formation is a driver of fibrosis. Orientation of the large fibres collagen and fibronectin are both regulated by periostin, which works in concert with tenascin-C (Kii et al. 2010). There is nothing known about the direct interaction between fibulin-1 and periostin.

### 1.6.9 Fibulin-1 and tenascin-C

Tenascin-C functions by altering the binding of cells on a fibronectin matrix and shares a binding site on the amino-terminal end of fibronectin with fibulin-1, the HepII region of fibronectin. (Williams and Schwarzbauer 2009). Cell signalling and matrix contraction are induced though this site and require binding of the heparin sulphate proteoglycan syndecan-4 (Midwood et al. 2006).

Syndecan-4 is an important mediator of fibroblast-matrix interactions. In syndecan-4 null mice, treatment with bleomycin resulted in a marked increase in myofibroblast recruitment and interstitial fibrosis compared to wild type controls (Jiang et al. 2010). Therefore, by competing for this binding site, both tenascin-C and fibulin-1 can influence fibrogenic cell adhesion and behaviour as both tenascin-C and fibulin-1 inhibit fibroblast spreading and cell-mediated contraction of a matrix (Williams and Schwarzbauer 2009) .

### 1.6.10 Fibulin-1 in embryonic morphogenesis and elastic fibre assembly

As previously discussed, fibulin-1 is a highly conserved ECM molecule that is essential during normal development (Singh et al. 2006). It is thought to play a role in stabilizing the ECM, as it colocalizes with elastin fibres during the first stages of embryogenesis (Visconti et al. 2003), the deposition of which occurs as one of the initial stages of development. Because elastic fibres are one of the two main determinants responsible for biomechanical properties of the lung (Faffe and Zin 2009), alterations in elastic fibre assembly affect lung function. Increased fibulin-1 deposition may alter elastic fibre assembly and hinder proper lung function.

### 1.6.11 The role of fibulin-1 in other diseases

Ultimately, as an ECM protein, and one that is essential to normal ECM formation, fibulin-1 plays an important role in diseases which involve ECM dysregulation. Fibulin-1 dysregulation in both the blood form and tissue form has been seen in a variety of diseases such as diabetes (Cangemi et al. 2011), preeclampsia (Liu et al. 2011), and synpolydactyly (Debeer et al. 2002). One common factor between these diseases is the disruption of the tissue architecture.

In the context of IPF, progressive disruption of the tissue architecture results in eventual lung failure and fibulin-1 may play a similar role in IPF pathogenesis.

### 1.6.12 Hypothesis

Fibulin-1 may be a biomarker of disease progression in patients with IPF as it is:

- Increased in fibrotic lung fibroblasts
- Increased in fibrotic tissue
- Induced by TGFβ1 in lung fibroblasts
- Increased in patients with fibrotic lung disease

Therefore, we hypothesize that fibulin-1 may play a role in disease progression by altering the 3D structure of the lung ECM as a consequence of its dysregulated deposition in the lung tissue. Elevated and progressive fibulin-1 deposition may result in gradual lung function impairment in patients with IPF (Figure 1.9).

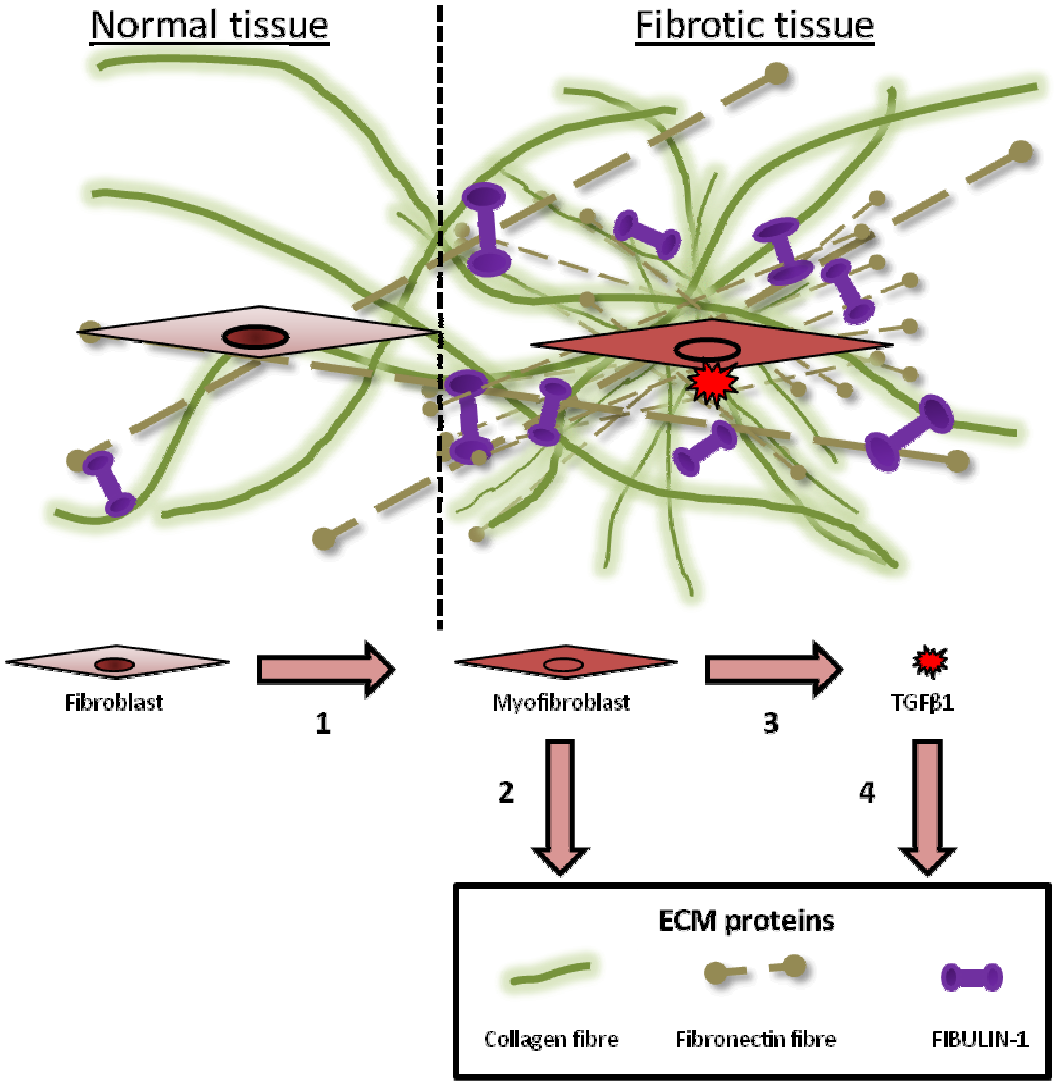



**Figure 1.9 The potential role of fibulin-1 in idiopathic pulmonary fibrosis**

Fibrotic lung tissue is different to normal lung tissue. (1) During fibrosis, lung fibroblasts are thought to take on a myofibroblast phenotype. (2) Part of this phenotype involves the increased deposition of extracellular matrix (ECM) molecules such as collagen and fibronectin. Other ECM proteins like fibulin-1 may also be increased. (3) Myofibroblasts also produce increased amounts of growth factors such as transforming growth factor-beta 1 (TGFβ1). (4) TGFβ1 is known to induce production of collagen and fibronectin by fibroblasts and it is possible that fibulin-1 is similarly upregulated.

## 1.7 Summary and Aims

IPF is a pulmonary disease that proceeds relentlessly with loss of the normal lung architecture and eventual respiratory failure, leading to death. Characterized by excessive ECM deposition, IPF is thought to be driven by a dysregulated wound repair process that involves activated fibroblasts. The pathogenesis of this disease remains unknown and there is a lack of mechanism-targeted treatments. Progression in IPF is also varied and more biomarkers of disease progression are needed.

Fibulin-1 is involved in matrix and elastic fibre formation and may be a biomarker of activated fibroblasts in the context of IPF. Mechanisms of fibrogenesis are known to be shared between pulmonary diseases and fibulin-1 may play a role in the pathogenesis of IPF. Fibulin-1 is produced by normal lung fibroblasts, but its expression in diseased fibroblasts has not been examined. In IPF, TGFβ1 induces excessive ECM production and TGFβ1 has been shown to induce fibulin-1 in the context of asthma, a disease which exhibits airway fibrosis, however, the effect of TGFβ1 on fibulin-1 in IPF has not been investigated.

The specific aims of this thesis are:

1. To examine the expression of fibulin-1 in patients with interstitial pulmonary fibrosis and to determine if fibulin-1 is a biomarker of disease progression in patients with IPF.
2. To compare the expression of fibulin-1 to other ECM proteins, that have been identified as dysregulated in the context of fibrotic interstitial lung disease, as biomarkers of disease progression in IPF.

3. To investigate the effect of TGFβ on fibulin-1 expression and production in fibroblasts derived from patients with IPF. The effect of TGFβ1 on the expression and production of other ECM proteins will also be investigated. In addition, the effect of TGFβ1 on fibroblast morphology will be examined.

# Chapter 2. **Fibulin-1 in Pulmonary Fibrosis**

## 2.1 Introduction

The heterogeneous group of more than 200 parenchymal diseases that comprise the interstitial lung disease (ILD) spectrum differ in their presentation, histopathology, clinical course and aetiology. ILDs are difficult for the non-specialist to diagnose, treat and monitor. For example, the ability to discriminate between chronic hypersensitivity pneumonitis (HP), an allergic lung disease also known as extrinsic allergic alveolitis, and idiopathic pulmonary fibrosis (IPF) is of particular concern due to their similarities in clinical presentation and stark difference in prognosis (Thomeer et al. 2004).

IPF is the most common of the idiopathic interstitial lung diseases and has the highest mortality (Demedts et al. 2001). Forty four percent of all IPF patients are expected to die within 5 years, compared with 33% of patients with connective tissue disease and 2% of patients with sarcoidosis, two other interstitial lung diseases (Demedts et al. 2001).

Treatment for ILDs are limited, differ widely between ILDs, and may be delayed as patients need to be referred to regional tertiary centres for the multidisciplinary diagnostic approach recommended by the guidelines (Raghu et al. 2011). In IPF, treatments have been largely unsuccessful (Raghu et al. 2013; Shulgina et al. 2013), mainly due to the fact that the underlying mechanisms of IPF are unresolved and there is no biomarker available to pinpoint individuals at risk of accelerated disease progression. Therefore, biomarkers in IPF that reflect states of active fibrogenesis in

the lung are needed to indicate those patients that will rapidly decline, in order to prioritize them for intensive management care and lung transplantation.

The large, fibrous extracellular matrix (ECM) proteins like collagen and fibronectin, are well-known to be increased in fibrotic conditions (Reiser and Last 1983; Muro et al. 2008; Araya and Nishimura 2010), but smaller, interlacing, ECM proteins can also play a key role. In their soluble form, altered ECM protein levels have been implicated in a variety of diseases such as cancer (Hellstrom et al. 2006) and mesothelioma (Robinson et al. 2003).

The ECM glycoprotein fibulin-1 binds to several key ligands involved in the fibrotic process, including fibronectin (Mattei et al. 1994; Tanaka et al. 1994; Godyna et al. 1995). Fibulin-1 is necessary during embryonic morphogenesis of several organs (Miosge et al. 1996; Cooley et al. 2008), and is essential for alveolar septa formation in the lung (Kostka et al. 2001). It is likely that there are common pathways between normal embryogenesis and fibrogenesis in many diseases with a fibrotic component (Araya and Nishimura 2010). The composition of the ECM is a known important and unifying factor in the pathophysiology of fibrosis, including lung fibrosis (Tschumperlin et al. 2012).

A number of ECM proteins also circulate in the blood. However, the utility of blood borne ECM proteins as serum biomarkers in IPF has not been well investigated (Richards et al. 2012; Vij and Noth 2012). In a recent review of peripheral blood biomarkers of importance in IPF, only one of the 13 described was an ECM protein, periostin (Vij and Noth 2012). This reflects our lack of knowledge of the contribution of particular components of the ECM to the disease pathology of pulmonary fibrosis.

Fibulin-1 is produced by lung fibroblasts (Roark et al. 1995) and has been shown to play a role in the pathophysiology of patients with asthma (Lau et al. 2010), a disease characterized in part by airway fibrosis (Royce et al. 2012). Therefore, it was reasoned that dysregulated fibulin-1 expression may be involved in lung diseases with more extensive fibrosis as mechanisms of fibrogenesis are likely to be shared (Kisseleva and Brenner 2008).

This study explored the role of fibulin-1 in disease pathogenesis and progression of IPF. Intitially serum and tissue fibulin-1 levels in patients with IPF and other ILDs were examined and related to lung function. Fibulin-1 production by lung fibroblasts derived from patients with IPF and from subjects without lung fibrosis were then measured. Lastly, whether serum fibulin-1 could serve as a biomarker was investigated and its prognostic utility assessed in several cohorts of IPF patients.

## 2.2 Methods

### 2.2.1 Patient Data

Due to the relatively low prevalence of ILDs within the local populations, this study involved collaborations with researchers in tertiary referral centres in Modena, Italy and San Francisco, USA. In addition, a researcher in Perth, Australia provided more samples of lung tissue from patients with IPF. The materials for this study were obtained from patients recruited from tertiary referral centres located in three different countries. Serum and tissue from Italy and San Francisco was obtained through collaboration with Professor Luca Richeldi and Dr. Stefania Cerri (University of Modena, Italy) and Professor Paul Wolters (University of California, San Francisco).

Tissue from Perth was obtained from Assoc. Prof. Cecilia Prele (University of Western Australia).

#### 2.2.1.1 Ethical Approval

Ethical approval for this study was obtained from each of the institutions involved, as detailed in Table 2.1. All participants provided informed patient consent.

**Table 2.1 Detailed ethical approval for the materials studied in this thesis**

| Institution | Location | # | Ethics Board | Sample collected |
|---|---|---|---|---|
| University of Sydney | Sydney, Australia | 2012/946 | University of Sydney Human Research Ethics Committee | Serum, tissue |
| Royal Prince Alfred Hospital | Sydney, Australia | HREC/10/RPAH/613 | Ethics Review Committee | Serum |
| Lung Institute of Western Australia | Perth, Australia | 2011-10-497 | Bellberry Human Research Ethics Committee | Serum, tissue |
| University of Modena | Modena, Italy | #74-08 and 31/12 | Comitato Etico Provinciale di Modena | Serum, tissue |
| University of California, San Francisco | San Francisco, USA | IRB#10-00198 | Laurel Heights Panel | Serum, lung lysates |

Australia: Consecutive patients referred to the Interstitial Lung Disease Clinic at the Royal Prince Alfred Hospital, Sydney were recruited and written informed consent attained. Diagnoses as determined by multidisciplinary review of patients included definite IPF (n=14), probable IPF (n=13), HP (n=11), and sarcoidosis (n=7). Other ILDs included non-specific interstitial pneumonia (NSIP) (n=3), connective tissue ILD (n=22), drug-induced ILD (n=1) and lymphangioleiomyomatosis (LAM) (n=2).

Healthy volunteers with no history of lung disease were also recruited (n=17).

Italy: Consecutive IPF patients referred to the Centre for Rare Lung Diseases at the University of Modena and Reggio Emilia were recruited and written informed consent attained. Diagnoses as determined by multidisciplinary review of patients included definite IPF (n=28) and other ILDs included lymphangioleiomyomatosis (n=2).

USA: Consecutive ILD patients recruited through the Nina Ireland Lung Disease program were recruited and written informed consent attained. Diagnoses as determined by multidisciplinary review of patients included definite IPF (n=17), HP (n=21), and sarcoidosis (n=5). Other ILDs included NSIP (n=1), and connective tissue ILD (n=4).

The total numbers of participants in each disease category are summarized in Table 2.2.

**Table 2.2 Numbers of participants in each of the diagnostic categories studied**

| Disease Category | Number of patients |
|---|---|
| No lung disease | 17 |
| Sarcoidosis | 12 |
| HP | 32 |
| "Other ILDs" | 35 total |
| • Connective tissue ILD | (26) |
| • NSIP | (4) |
| • LAM | (4) |
| • Drug induced ILD | (1) |
| IPF | 72 |

Lung Function Measurements: Demographic information including age, gender, body mass index (BMI) and smoking history were collected. Baseline lung function measurements taken included pre-bronchodilator percent predicted forced vital capacity (FVC%), percent predicted forced expiratory volume in 1 second ($FEV_1$%), percent predicted diffusing capacity of carbon monoxide ($DL_{CO}$%) and percent

predicted total lung capacity (TLC%) (Miller et al. 2005).The Composite Physiologic Index (CPI) was calculated as previously published (Wells et al. 2003). Follow up FVC% and $DL_{CO}$% were collected at 365 days (±1) when available.

### 2.2.2 Definition of progression

Patients with IPF were followed up for a minimum of one year (365 ± 1 day) after blood draw. A progression event was defined as any of the following occurring within the first one-year follow up period, ≥10% relative fall in FVC%, ≥15% relative fall in $DL_{CO}$%, or death, as previously published (Ryerson et al. 2013).

### 2.2.3 Serum collection

Whole blood from patients and healthy volunteers in Sydney was collected, processed and stored by Jade Jaffar. Methods for whole blood processing from all centres were as follows:

Whole blood was isolated from patients using sterile venepuncture technique by a qualified phlebotomist into serum Vacutainer (Becton Dickinson, USA cat #367958). The containers were inverted five times and left upright to clot for a minimum of 30 minutes at room temperature (approximately 22-25°C). Samples were then centrifuged at 14,000 x g for 10 minutes and the serum fraction was aspirated with a sterile pipette. Aliquots of 200 – 500µL of serum was stored at -80°C until testing.

### 2.2.4 Tissue collection

Australia: Lung tissues from patients with IPF, who had provided written informed consent, were obtained from explanted lung following lung transplantation at St. Vincent's Public Hospital, Sydney (n=5; 2 samples per patient) or from diagnostic

biopsies from Perth (n=7; 2-6 samples per patient). Where permission had been provided by the next of kin for the lungs to be used for research purposes, normal human lung tissue was obtained from healthy transplant donors' lungs that were deemed not suitable for transplantation (n=5) .

Italy: Excess tissue following diagnostic surgical lung biopsies were obtained from patients with IPF who had given permission for their tissues to be used for research (n=6; 2-6 samples per patient).

USA: Whole lung lysates extracted from explanted lung following transplantation. Where permission had been provided for the tissue to be used for research were stored at -80°C until testing (n=8). The method for preparation of lung lysates is found below.

#### 2.2.4.1 Tissue processing

Tissue samples used for immunohistochemistry were processed as follows. Following removal from the patient, the tissues were submerged in 10% neutral buffered formalin solution (Sigma Aldrich, Sydney, Australia) to prevent post mortem decomposition. Tissues were then submerged in graded alcohol baths (from 70% alcohol to 100% alcohol) until all water had been removed. Finally, the samples were submerged in xylene and embedded in paraffin wax for sectioning.

Whole lung lysates were a gift from Dr. Paul Wolters (University of California, San Francisco) and were prepared as follows: Lung tissue was directly snap-frozen in liquid nitrogen immediately after harvest. Samples were stored at –80°C until used for experiments. For immunoblot experiments, frozen lung tissue was pulverized using a stainless steel tissue pulveriser (Fisher Scientific, USA) pre-cooled in liquid nitrogen

and was immediately lysed in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Sigma-Aldrich, USA) running buffer and analysed as described below.

### 2.2.5 Cell isolation

Primary parenchymal fibroblasts were isolated from 7 patients diagnosed with non-small cell carcinoma and 8 patients with IPF. Human distal parenchymal fibroblasts were isolated from lung tissue obtained from donors undergoing resection for either thoracotomy or transplantation. For all experiments in this thesis, cell cultures at less than 6 passages are used. Demographic information for donors of tissue is found in Table 2.8.

Tissue from distal parenchyma was minced into 1-2mm$^3$ pieces and placed into sterile Hanks Buffered Saline Solution (Hanks) (Sigma Aldrich, Sydney Australia) and centrifuged for 5 minutes at 1000rpm. After aspiration of the supernatant, the tissue pellet was resuspended in media containing 10% (vol/vol) foetal bovine serum (FBS) with 2% Penicillin-Streptomycin (Invitrogen) in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) and plated into tissue culture grade plastic flasks (BD Biosciences, North Ryde, Australia).

### 2.2.6 Cell culturing

Cells were passaged using 0.4% trypsin-EDTA (Thermo Scientific, Melbourne, Australia): a confluent monolayer of cells from a single flask was washed with Hanks and then incubated with 5 ml 0.4% trypsin-EDTA (Thermo Scientific, Melbourne Australia) for up to 5 minutes at 37oC, 5% CO2. The trypsin was then inactivated with double its volume of 5%FBS/DMEM. The cells were then centrifuged for 5 mins at 1000 x g and resuspended in 6mL 5% FBS/DMEM supplemented with 1%

Pencillin-Streptomycin (anti-mycotic) (growth media) and divided into three new 175 $cm^2$ sterile culture flasks each containing 8mL growth media.

Passages of cells between 3 and 5 were used in all experiments. All cultures tested negative for the presence of mycoplasma before use in experiments.

### 2.2.7 Cell experimentation

Primary fibroblasts were seeded in 6-well plates at 1 x $10^4$cells/$cm^2$ in 5%FBS/1% antibiotics/DMEM for 72 hours and then quiesced in 0.1% FBS/1% Pencillin-Streptomycin /DMEM (quiescing media) for 24 hours. Fresh quiescing media is then added for a further 72 hours.

#### 2.2.7.1 RNA collection

Total RNA was collected using the BIOLINE mRNA Isolate Miniprep kit (Bioline, London, UK). Cells were seeded in 6-well plates as previously described and the supernatant was removed and discarded. Cells were lysed with 450µL of Lysis Buffer and incubated for 3 minutes at room temperature. Cell lysates were collected and placed into the 1.5mL spin column R1 placed inside a collection tube. After spinning at 10,000 x g for 2 minutes at room temperature, the filtrate was saved and transferred to a new spin column R1. 450µL of 70% ethanol was added to the filtrate and spun again at 10,000 x g for 2 minutes at room temperature. The filtrate was then discarded and 500µL of wash buffer AR was added. The column was spun again at 10,000 x g at room temperature for 1 minute and the filtrate was discarded. 700µL of wash buffer BR was added and the column was spun for 1 minute at 10,000 x g at room temperature, the filtrate was discarded and then the column was spun again at 10,000 x g for 2 minutes. Finally, the column was placed in a fresh RNA-free microtube and 30µL of RNase-free water was added. Following 1 minute incubation at room

temperature the RNA was collected by spinning the tube at 6,000 x g for 1 minute at room temperature. The concentration of the RNA was measured using a Nanodrop 2000 (Thermo Scientific, Melbourne, Australia).

The Nanodrop 2000 is a spectrophotometer that measures the light diffraction in a 1µL sample of RNA isolate using a narrow beam of light. The amount of light that travels through the sample corresponds to the amount of RNA present.

RNA was stored at -80°C until conversion to cDNA.

#### 2.2.7.2 Cell supernatant and cell lysate collection

Cells were seeded in 6-well plates as previously described. Cell culture supernatants were collected into microtubes and the remaining cells are immediately washed twice in cold sterile phosphate buffered saline (PBS, pH 7.2).

On wet ice, total cellular protein extracting buffer was added. Extraction buffer contains 20mM Tris, pH 7.4, 150mM NaCl, 1mM $Na_2EDTA$, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% protease inhibitor cocktail set III (Millipore, USA) and 1mM phenylmethylsulfonyl fluoride (PMSF) (Amresco, Solon, OH, USA).

Lysates are then collected and centrifuged at 4°C/14,000g for 5 mins to pellet debris. The cell debris-free fraction was then aspirated and aliquoted into a fresh microtube. Cell supernatants and lysates were stored at -20°C until analysis. This extraction buffer is also used as a diluent in immunoblot experiments.

### 2.2.8 Real-time reverse transcription polymerase chain reaction (QPCR)

#### 2.2.8.1 mRNA to cDNA conversion

PhD candidate Alen Faiz isolated and converted the mRNA to cDNA specific to this chapter. All further experiments were performed and analysed by Jade Jaffar.

500 ng of mRNA was converted to cDNA using the M-MLV reverse transcriptase (Invitrogen, USA cat# 28025-013). On ice, 500 ng of the mRNA isolate was added to 1 µL of random primer (Biolabs cat#S1230S). The random primer was a mixture of hexamers (a 6 nucleotides fragment of cDNA) that was used to start the cDNA conversion. To this, 1 µL of 10mM dNTPs (Invitrogen) was added and DNA-free water was added to make the final volume 12 µL. This mixture was heated for 5 minutes at 65°C and then immediately chilled on ice.

Next, 4 µL of 1$^{st}$ strand buffer (Invitrogen, cat#Y002321), 2µL of 0.1M dithiothreitol (DTT)(Invitrogen, cat#Y00147), and 1µL of ribonuclease inhibitor (Invitrogen, cat#10777-019) was added to the mixture and incubated for 2 minutes at 37°C. Finally, 200 units (1 µL) of M-MLV reverse transcriptase was added. The mixture was incubated at 25°C for 10 mins, then at 37°C for 50 minutes and finished with a 15 minute incubation at 70°C to stop the reaction. cDNA was then stored at -20°C until use in QPCR.

#### 2.2.8.2 Measurement of fibulin-1 mRNA levels

A commercially available QPCR primer pair specific for human fibulin-1 was obtained from Invitrogen (Hs_00243545_m1). Quantitative analysis of fibulin-1 expression was performed using human 18S rRNA (Invitrogen) as the endogenous control. All samples were kept on ice during experiment set up and every sample was tested in triplicate. To each 3.3µL sample of cDNA, 33µL of TaqMan Universal Master Mix (Invitrogen), 3.3µL of fibulin-1 primer, 3.3µL of 18S primer and 23.1µL of DNA-free water was added. In a 96-well PCR plate (Invitrogen) 20µL of this

solution was added in sequential wells. As a negative control, three wells that contained the master mix, primers, and water, but without cDNA was included.

Thermal cycle conditions were 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (Johnson et al. 2006). QPCR was performed using the StepOne Plus detection system and the data were collected and analyzed using StepOne software (Applied Biosystems, Melbourne, Aus). The relative abundance of mRNA was calculated by using the ΔΔCt method and the results were normalized against the 18S rRNA levels.

### 2.2.9 Immunoblotting

Placental fibulin-1 was donated by Dr. Scott Argraves (Medical University of South Carolina) and was purified as published (Twal et al. 2001). Placental fibulin-1 was reconstituted in sterile PBS (Invitrogen) and was used as a positive control in immunoblot experiments.

Cell-derived fibulin-1 was also donated by Dr. Scott Argraves. The fibrosarcoma cell line HT-1080 (Rasheed et al. 1974) was transfected with the fibulin-1 gene in their lab. This cell line was chosen as it does not normally produce the fibulin-1 protein (Godyna et al. 1995; Xie et al. 2008).

Fibulin-1 transfected HT-1080 cells were grown in growth media supplemented with 300µg/mL G418 antibiotic (Invitrogen) as a selection agent. This maintained the selection pressure an ensured that all the cells present contain the plasmid with the fibulin-1 gene. HT-1080 cell supernatants and cell lysates were collected from tissue culture flasks as previously described (2.2.6) and used as positive controls in immunoblot experiments.

Whole lung lysates, fibroblast cell lysates, fibroblast supernatants and sera were separated by SDS-PAGE. For whole lung and cell lysates, total protein was measured by Bicinchoninic Acid (BCA) assay (Sigma Aldrich, Melbourne, Australia cat #BCA1). The separating gel was made of 25% Tris-SDS buffer (pH 8.8), 0.1% w/v N,N,N‘‘,N‘‘-Tetramethylethylenediamine (TEMED) (Sigma) , 1% w/v ammonium persulfate (APS) (Sigma), and 25% v/v Acrylamide:Bis solution (40% 37:1 Acrylamide:Bis-acrylamide) (Bio-Rad) in distilled water. The stacking gel consisted of 25% Tris-SDS buffer (pH 6.8), 0.1% w/v TEMED, 1% w/v APS, and 10% v/v Acrylamide:Bis solution in distilled water.

Using the mini-PROTEAN gel casting equipment (Bio-Rad), the separating gel was poured and flattened using 500µL of 100% butanol to ensure an even polymerisation across the gel. After letting the gel set for at least 2 hours at room temperature, the butanol was decanted and the gel was washed twice with distilled water and the stacking gel was added along with 15-well combs (Bio-Rad) and allowed to set for a further 30 minutes.

A 5X loading dye was made consisting of 0.312M Tris-HCl, 5% SDS, 0.5M DTT, and 50% glycerol in distilled water and then diluted with sample/lysis buffer at 1:5 dilution for use. The pre-stained molecular weight marker (ranging between 10 – 250kDa) was sourced from Bio-Rad (USA).

The samples were diluted as follows:

| Sample | Quantity of sample | Volume of extraction buffer | Volume of 5X loading dye |
|---|---|---|---|
| Serum (pre-diluted 1:10 with sterile PBS) | 8µL | 72µL | 20µL |
| Whole lung lysates | 0.15µg/µL | As needed* | As needed* |
| Fibroblast cell lysates | 0.15µg/µL | As needed* | As needed* |
| Fibroblast cell supernatants | 6µL | none | 24µL |
| Placental fibulin-1 | 0.5ng/µL | As needed* | As needed* |
| HT-1080 cell lysate | 0.15µg/µL | As needed* | As needed* |
| HT-1080 supernatant | 3µL | 3µL | 24µL |

*As needed, the volumes of lysis buffer and dye were adjusted to provide a final concentration of the sample as listed.

Samples diluted in lysis buffer and/or extraction buffer (10µL/well) was added to each well and run in SDS electrophoresis tank buffer which consisted of 0.3% w/v Tris-Base, 1.44% w/v glycine and 0.1% w/v SDS in distilled water (pH 8.5). A Bio-Rad power pack 300 was set at 125 V and the gel was run for approximately 90 minutes.

Proteins were transferred to a 0.45µm pore size polyvinylidenedifluoride (PVDF) membrane using a Bio-Rad trans-blot tank filled with 10% methanol in SDS electrophoresis buffer. Bio-Rad power pack was set at 25 V for 1.5 hours. Membranes were blocked with 5% skim milk/2% BSA/TBS-Tw for 1 hour at room temperature, and rinsed quickly with TBS/Tw before being probed with primary antibody to fibulin-1 (#SC25281, Santa Cruz Biotechnologies, USA) diluted to a concentration of 0.2 µg/mL in 2% BSA/TBS-Tw for two hours at room temperature or overnight at 4°C.

Membranes were washed in three changes of TBS-Tw for 5 minutes each at room temperature before being incubated with horseradish peroxidase-conjugated secondary anti-mouse antibodies (Dako, USA) diluted to 0.1µg/mL in 2% BSA/TBS-Tw for one hour at room temperature. Membranes were again washed in three changes of TBS-Tw for 5 minutes at room temperature before visualization was carried out by incubating the membranes in enhanced chemiluminescence (ECL) western blot detection reagent (Millipore, USA) for one minute. A Kodak Image Station 4000MM camera was used to capture images and analysis was performed using Carestream Molecular Imaging software (v. 5.3.3.17476 Carestream Health Inc. 1994-2011).

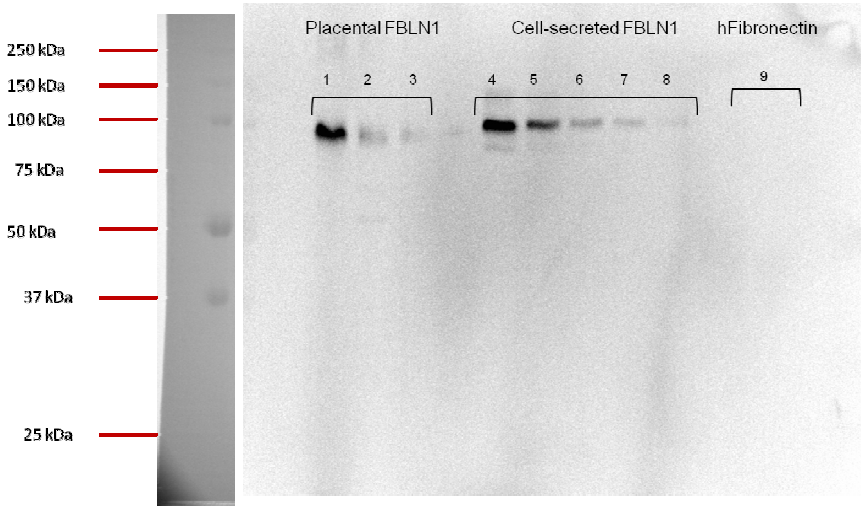
Membranes loaded with whole lung or cell lysates were then stripped by incubating the membranes in 2% SDS/Tris-HCl (pH 6.8) supplemented with freshly added 0.8% β-mercaptoethanol (Sigma) for 15 minutes at 50°C. Membranes were washed in 3 changes of TBS-Tw as before and then blocked again using 5% skim milk/ 2% BSA/ TBS-Tw for another hour at room temperature.

Membranes were incubated at room temperature for 45 minutes with anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Millipore) diluted to 0.04µg/mL in 2% BSA/TBS-Tw. Membranes were washed in three changes of TBS-Tw for 5 minutes each before being incubated with horseradish peroxidise-conjugated secondary anti-mouse antibodies (Dako, USA) diluted to 0.02µg/mL in 2% BSA/TBS-Tw for 30 minutes at room temperature. Membranes were again washed in three changes of TBS-Tw for 5 minutes before visualization was carried out as before. Image capture and analysis were carried out as described above.

### 2.2.10 Specificity of fibulin-1 antibody

There were no previous reports in the literature of publications involving the use of the SC25281 monoclonal antibody and western blotting as a method for the quantification of fibulin-1. Therefore, initial experiments were carried out to ensure specificity and sensitivity of the antibody for use in this study (Figure 2.1). Fibulin-1 was discovered as a binding partner for fibronectin (Argraves et al. 1989) and therefore it was important to ensure that the fibulin-1 specific antibody did not cross react with fibronectin. Fibronectin is also present in high concentrations in the blood (Lafuma et al. 1987) and could affect the measurements. Anti-fibronectin antibody (#MAB1935, Chemicon, USA) and plasma purified fibronectin protein (#FC010, Millipore, USA) were used as negative controls. By assaying the samples at 3-5 concentrations, a standard curve was drawn indicating that there was a linear relationship between the concentration of fibulin-1 and the resulting band intensity and that this method could be used to quantify the level of fibulin-1 in samples (Figure 2.1).

Importantly, Figure 2.2 shows that there was no fibronectin contamination in either of the positive fibulin-1 control materials used in this study. Finally, all quantification of fibulin-1 (whether in serum or tissue) was done without knowledge of the patient's corresponding lung function measurements in order to ensure the data were analysed in a blinded manner.




A

| Lane | Dilution | Net Intensity | FBLN1 Conc | Sum Intensity | Mean Intensity | Interior Area (pixels) |
|---|---|---|---|---|---|---|
| Placental FBLN 8ng | 1 | 270143 | 8 | 1425983 | 157.9162 | 9030 |
| Placental FBLN 4ng | 2 | 36226 | 4 | 1192066 | 132.0117 | 9030 |
| Placental FBLN 2ng | 4 | 17684 | 2 | 1182554 | 130.9584 | 9030 |
| HT1080-C 6ug | 2 | 252986 | 6 | 1453976 | 161.0162 | 9030 |
| HT1080-C 3ug | 4 | 115512 | 3 | 1325532 | 146.792 | 9030 |
| HT1080-C 1.5ug | 8 | 47804 | 1.5 | 1212674 | 134.2939 | 9030 |
| HT1080-C 0.75ug | 16 | 33435 | 0.75 | 1171215 | 129.7027 | 9030 |
| HT1080-C 0.38ug | 32 | 11237 | 0.38 | 1149017 | 127.2444 | 9030 |

B

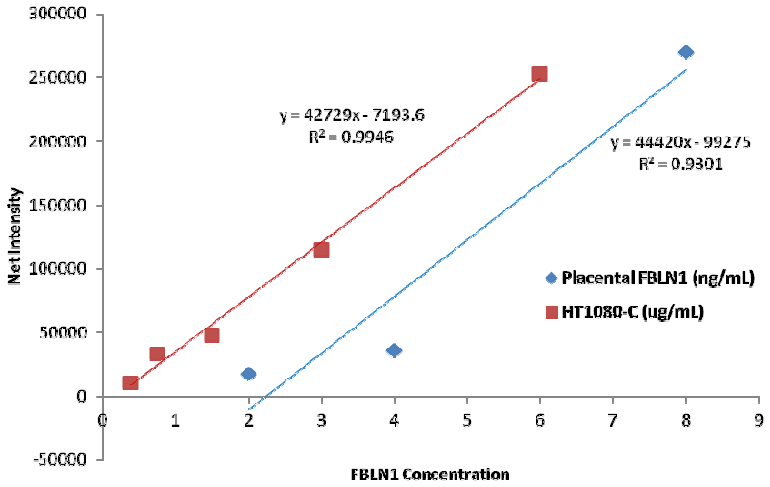



**Figure 2.1 The sensitivity and specificity of the fibulin-1 antibody**
A representative immunoblot showing fibulin-1 (FBLN1) protein purified from human placenta (lanes 1-3), recombinant fibulin-1(C-isoform) isolated from transfected human fibrosarcoma cell line (HT-1080) (lanes 4-8), and purified human plasma fibronectin (hFibronectin, #FC010, Millipore, USA)(lane 9) as probed by anti-fibulin-1 antibody (#SC25281, Santa Cruz Biotechnologies, USA). Protein size ladder is shown on the left.
Cell-secreted FBLN1 is the cell-free culture supernatant from HT-1080 cells that have been transfected with the FBLN1(C-isoform) gene.(A) Densitometric analysis was performed on the immunoblot shown. A dilution of 1 indicates an undiluted sample. Fibulin-1 concentration (FBLN1 conc) was measured by BCA assay. Net, Sum, and Mean Intensity of each band was analysed by Kodak Image Station 4000MM (Carestream Molecular Imaging Software v. 5.3.3.17476). The Interior Area indicates the number of pixels that is analysed for each sample. The Sum Intensity indicates the total intensity of each pixel in the interior area. The Mean Intensity is the average intensity of the interior area. The Net Intensity is the sum intensity minus the intensity of the background of the blot.
(B) Net intensity of each band is then plotted against the FBLN1 concentration and a linear regression is performed.

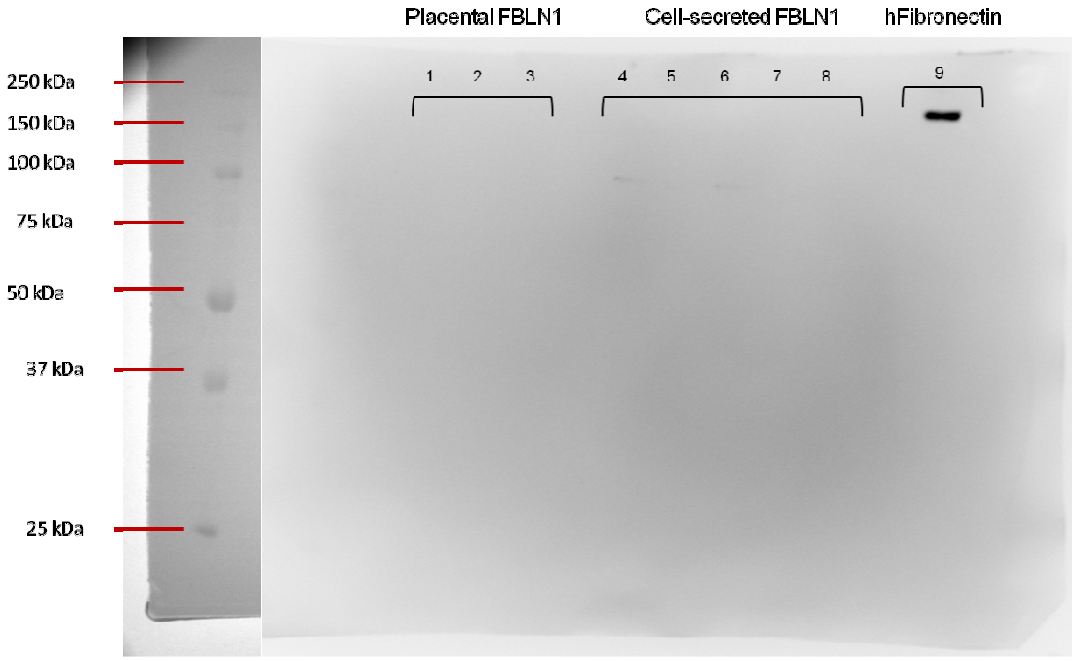



**Figure 2.2 Purity of fibulin-1 positive controls were investigated by probing with an antibody to human fibronectin**

A representative immunoblot showing fibulin-1 (FBLN1) protein from placenta (lanes 1-3), from a fibulin-1(C-isoform) transfected human fibrosarcoma cell line (HT-1080) (lanes 4-8), and purified human plasma fibronectin (hFibronectin, #FC010, Millipore, USA)(lane 9) as probed by anti-fibronectin antibody (#MAB1935, Chemicon, USA). Protein size ladder is shown on the left. Net, Sum, and Mean Intensity of each band was analysed by Kodak Image Station 4000MM (Carestream Molecular Imaging Software v. 5.3.3.17476).

Concentration of the proteins loaded were as follows:

Lane 1 8ng/mL, Lane 2 4ng/mL, Lane 3 2ng/mL, Lane 4 6ug/mL, Lane 5 3ug/mL, Lane 6 1.5ug/mL, Lane 7 0.75ug/mL, Lane 8 0.38ug/mL, Lane 9 100ng/mL

Lastly, the level of fibulin-1 in serum samples at the same concentration were compared (Figure 2.3). Serum fibulin-1 contained 4 bands. Fibulin-1 was considered to be 100kDa as this is the size of the protein that was first identified as a binding partner of fibronectin (Argraves et al. 1989). The other bands may be fragments of fibulin-1 or non-specific binding of the antibody to other proteins which have not been identified in this thesis. To our knowledge, this is the first time that serum fibulin-1 has been quantified in this manner and there is no evidence in the literature of other sizes of fibulin-1 in the serum.

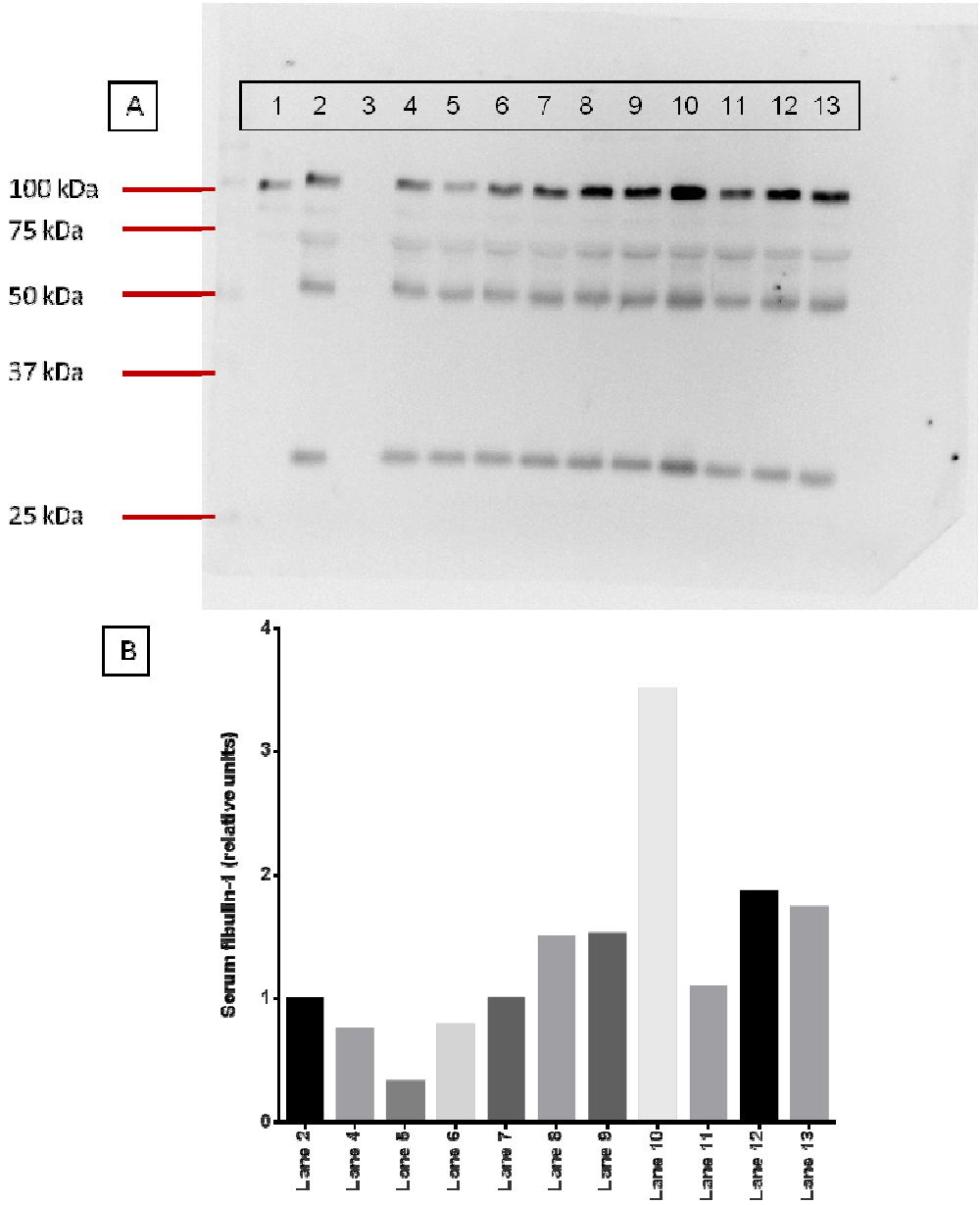



**Figure 2.3 Serum levels of fibulin-1 quantified by western blot.**
(A) A representative immunoblot of fibulin-1 in serum samples. In lane 1, placental fibulin-1 (5ug/mL) loaded as a positive control. Lane 2 contained the serum sample used for relative quantification of fibulin-1 and is diluted 1:500. Lane 3 contained 100ng/mL human plasma fibronectin as a negative control. Serum from ten independent patients was diluted 1:500 and loaded in lanes 4-10. Densitometric analysis was performed using Kodak Image Station 4000MM (Carestream Molecular Imaging Software v. 5.3.3.17476). Fibulin-1 is 100kDa.

(B) The net intensity of the 100kDa band of lane 2 was set to 1. The net intensities of each of the patient samples were then calculated relative to the net intensity of lane 2. Fibulin-1 level was reported in relative units.

### 2.2.11 Optimization of normalization protocol for cell supernatants

There is evidence in both the literature (Ramos et al. 2001) and from work previous completed in our laboratory (Havryk, PhD thesis 2005) that fibroblasts from patients with IPF have lower levels of proliferation compared to fibroblasts from subjects without lung disease. Therefore it was important to take cell number into account (which has been referred to as normalisation) when comparing the level of fibulin-1 in cell culture supernatants.

This experiment was designed to investigate the effect of different methods of cell number normalisation and 4 cell lines were determined to be enough to address this question. Fibroblasts from patients with IPF (n=2) and subjects without IPF (n=2) were grown in 5% FBS/ DMEM/1% Penicillin-Streptomycin for 72 hours described in Chapter 2.2.6 and supernatants were harvested as described in Chapter 2.2.7.2. Duplicate wells were harvested for estimations of cell numbers by (A) manual cell counting, (B) commercially available DNA-binding assay CyQuant (#C7026, Invitrogen, USA) (Marinkovic et al. 2012), and by (C) measurement of the total protein concentration of the cell monolayer as described in the Methods 2.2.9. Briefly, plates used for manual cell counting were washed and cells were detached using trypsin as described in Chapter 2.2.6. Total cells were stained with haematoxylin (Sigma Aldrich, Melbourne Australia) and the ones dye excluded were counted using a haemocytometer. The average of 10 large squares was calculated.

The fibulin-1 level in the cell supernatant as measured by immunoblot was then normalized to each of the three cell number estimations (Figure 2.4). There was no difference between the level of fibulin-1 when normalized to either manual cell

number (Figure 2.4A), Cyquant estimation (Figure 2.4B), or to the concentration of the cell monolayer (Figure 2.4C).

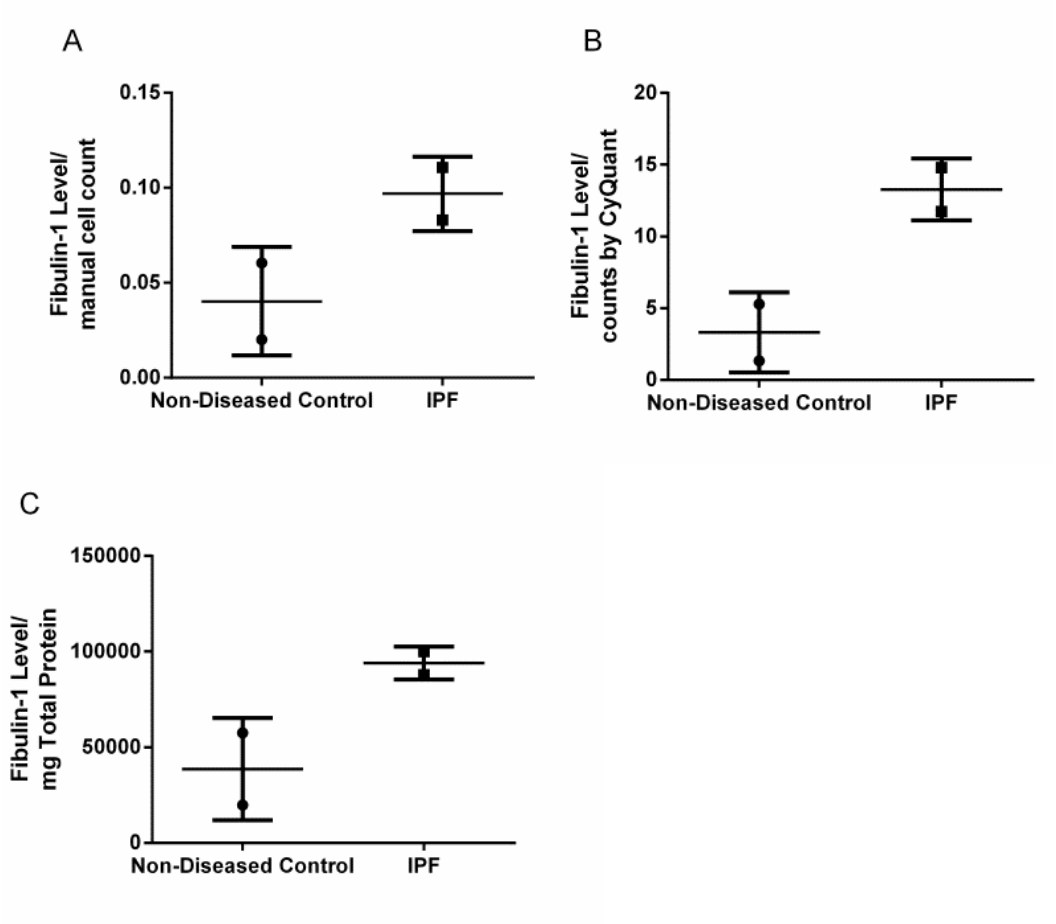



**Figure 2.4 The effect of normalization to different cell number estimations on fibulin-1 level.**
Fibroblasts from four patients were grown in 5% FBS/DMEM for 72 hours and supernatants were harvested. Fibulin-1 level was measured by immunoblot. Total cell numbers were estimated using (A) manual cell counts as performed by haemocytometer, (B) commercially available CyQuant assay (#C7026, Invitrogen, USA), and (C) the total protein concentration as measured by BCA assay.
BCA bicinchroninic acid, IPF idiopathic pulmonary fibrosis

Subsequently, for measurements of fibulin-1 level in fibroblast supernatants, equal volumes of supernatant were loaded and final fibulin-1 levels are adjusted for the total protein concentration of the respective confluent cell monolayer lysate as measured by BCA and described in the literature ((Quesnel et al. 2008; Quesnel et al. 2010)).

### 2.2.12 Immunohistochemistry

Five micrometre thick paraffin sections from formalin-fixed tissue were directly stainedfor fibulin-1 and in parallel with the matching isotype control, mouse IgG2a (Dako, Sydney, Australia). The concentration of the primary antibodies that were used are found in Table 2.3.

**Table 2.3 Concentrations of the primary antibodies used for immunohistochemistry**

| | Source | Final concentration |
|---|---|---|
| Fibulin-1 antibody | Santa Cruz (cat# 25281) | 0.01µg/mL |
| Mouse IgG2a antibody | Dako | 0.01µg/mL |

Slides were deparaffinised by incubating in 2 baths of xylene for 5 minutes at room temperature. This was followed by 2 minute incubations each in 2 baths of 100% ethanol, 95% ethanol, 70% ethanol and 50% ethanol before being left standing in distilled water for the final 2 minutes or up to overnight.

No antigen retrieval was used. Antigen retrieval is a technique employed to "unmask" sites for antigen binding that may be blocked by the fixation process. Slides were then blocked for one hour at 37°C in 10% normal goat serum diluted in pH 7.4 Tris buffered saline (0.3% Tris, 0.8% NaCl, 0.02% KCl) supplemented with 0.05% tween-20 (Sigma) and 1% BSA (1% BSA/ TBS-Tw). The blocking solution prevents non-specific binding of the primary antibody and was removed by tapping the slide once on paper towels. The fibulin-1 antibody was diluted in 1% BSA/TBS-Tw and 50 – 200 µL of the solution was loaded onto each slide. Slides were incubated at 37°C for another 2 hours.

Slides were then washed twice in TBS-Tw for 5 minutes each and oxidised in 3% hydrogen peroxide for 5 minutes at room temperature. This prevents the endogenous peroxidise activity in the tissue from affecting the secondary reagent. Slides were again washed twice in TBS-Tw for 5 minutes each and 50 to 200 µL of the secondary reagent (Dako cat#K4006) was added for 30 minutes at room temperature.

After incubation with DakoEnVision secondary reagent, areas that were positive for the antibody were visualized by incubating in diaminobenzidine (DAB) (Dako cat#K3467) for 2 minutes. This dye was diluted 1 drop per mL of substrate for use and turned brown when in contact with the horseradish peroxidase (HRP) enzyme that was conjugated to the anti-mouse secondary antibody. The reaction was stopped once the slides were dipped in distilled water. Sections were counterstained by incubating in Mayer's haematoxylin (Sigma cat#MHS1) for 3 minutes. Slides were then washed briefly in tap water, taken backwards through the graded alcohols to finish in xylene and coverslipped with the xylene-based mounting medium DPX (Sigma cat# 44581).

Serial sections were also stained using standard Masson's Trichrome in order to quantify total collagen levels. Masson's Trichrome staining was performed by Dr. Rema Oliver, University of New South Wales. Following deparaffinisation as described above, slides are incubated in Weight's haematoxylin for 20 minutes at room temperature. Slides were washed in running tap water for 5 minutes before incubation with 0.5% acid fuchsin solution and then again washed briefly in tap water. Slides were then incubated for 25 minutes in 1% phosphomolybdic acid and rinsed in tap water before incubating for 3 minutes in 2% Light Green Counterstain solution. Slides were washed with tap water and incubated for 1 minute in 1% acetic acid before being dehydrated in the graded alcohols and mounted in DPX as before.

Collagen stained green while cell nuclei were a blue/black. Cytoplasm, muscle or erythrocytes were red.

### 2.2.13 Image Capture

A majority (75%) of the image capture of stained tissue sections was performed by undergraduate student Sofia Unger as part of a student research project, under the tutelage of Jade Jaffar. All further experimentation and analysis was performed by Jade Jaffar.

Western blot images were captured using a Kodak Image Station 4000mm camera and analysed using Carestream Molecular Imaging software (v. 5.3.3.17476 Carestream Health Inc. 1994-2011). Immunohistochemisty images were taken at 20X magnification using an Olympus BX60 microscope (North Ryde, Australia). 20 consecutive (minimally overlapping) images of each section were randomly (not focused on any particular structure) taken using a DP71 camera with Kohler illumination and it was ensured that the entire surface of the section was included in the final analysis. Pictures of all sections that were stained in the same manner were taken on the same day to maintain identical light settings. Two to six sections from each patient were imaged.

### 2.2.14 Densitometric analysis

The images of sections stained for fibulin-1 were analysed by the open source software ImageJ (http://rsb.info.nih.gov/ij/ accessed June 2011) (Busquets et al. 2010). First, each image was colour deconvoluted (Ruifrok and Johnston 2001; Lesack and Naugler 2012) using an algorithm and the plugin vector H&E DAB (http://www.dentistry.bham.ac.uk/landinig/software/cdeconv/cdeconv.html, accessed

July 2013). The resulting brown stain was further investigated and a threshold of positive staining was set manually by examining 5 random images of sections from 5 independent non-diseased control patients. This threshold was then applied to every image. The algorithm then calculated the positively stained area as a numerical value and adjusted for the total positively stained area in the image. The resultant mean value took into account any compression of the tissue during processing as well as the increased tissue mass in fibrotic lesions (Haafiz et al. 2011).

Similarly, images of sections stained with Masson's trichrome were colour deconvoluted using an algorithm and the plugin vector Masson trichrome (http://www.dentistry.bham.ac.uk/landinig/software/cdeconv/cdeconv.html, accessed July 2013). The resulting green stain was quantified and reported as the percentage area of collagen.

Each of the 20 image values in each analysis set were then averaged to obtain the average staining density for each tissue section.

Finally, the multiple samples from each patient were averaged together to give the final fibulin-1 level or percentage area of collagen per patient. A sample of an image used for analysis is seen in Figure 2.5.

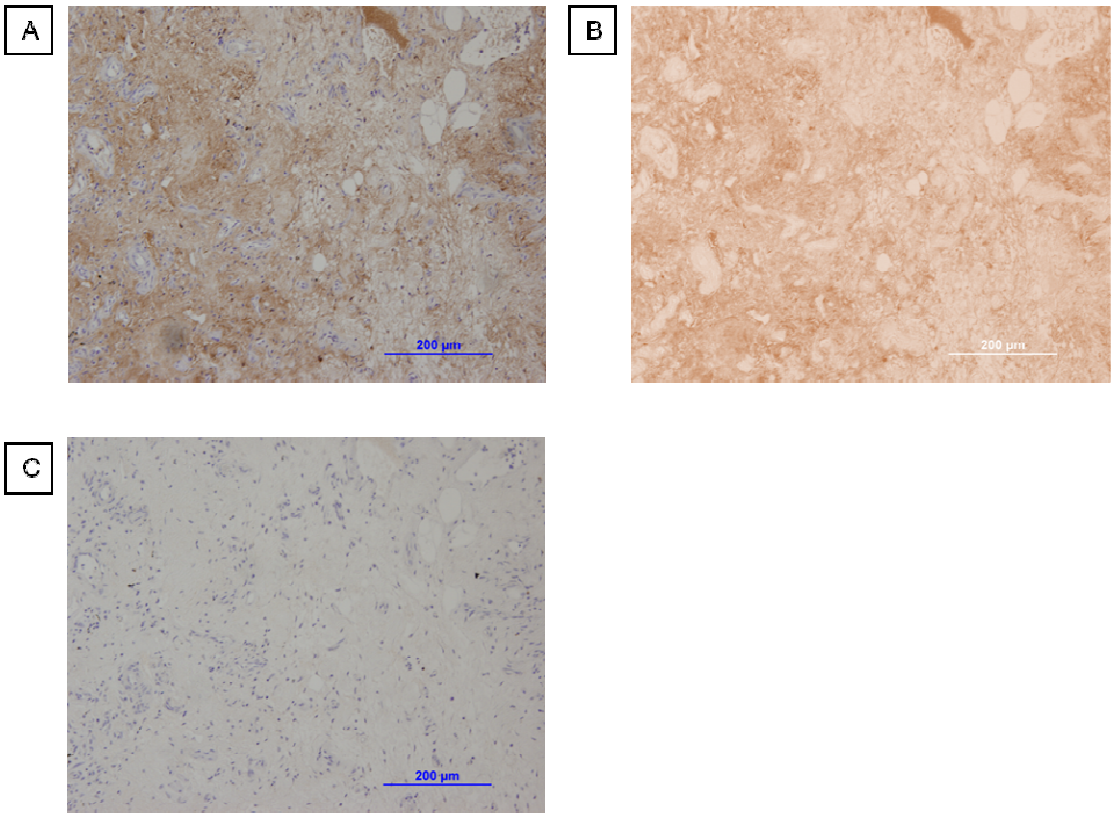



**Figure 2.5 Quantification of fibulin-1 level by computer-based image analysis**
(A) A single image of a tissue section stained for fibulin-1 (brown) and counterstained with haematoxylin (blue). (B) A computer algorithm separates the brown stain from the blue stain. The level of brown staining can then be quantified and in this case measures 131 units. (C) A sequential tissue section is stained with the isotype control for fibulin-1 and is used for quality control purposes. The level of brown staining in the isotype control should not be greater than that of the fibulin-1 stained section. Details regarding methods can be found in 2.2.14

### 2.2.15 Statistical analysis

To determine the repeatability and the reliability of the measurements, the coefficient of repeatability and intraclass correlation coefficient were calculated (Bland and Altman 1996).

Duplicate serum samples from 17 subjects were tested on two separate occasions and the coefficient of repeatability was 1.48 units of fold difference. This indicates that the same serum tested the first time would be expected to differ by more than 1.48 fold on the second test only 5% of the time. The intraclass correlation coefficient was 0.91 (95% CI 0.77 to 0.97) indicating that there was a high degree (>0.6) of reproducibility of the serum measurements.

Graphs were made using GraphPad Prism 6 Software for Windows (Version 6 GraphPad Software Inc. 1992-2007, Redmond, USA). Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) (Version 21 IBM Corporation 1989-2012, Armonk, USA).

Characteristics of the subjects and spirometric results were summarized with the use of descriptive statistics.

Distributions of serum, parenchyma and fibroblast levels of fibulin-1, and lung function parameters were tested for normality using the Kolmogorov-Smirnov test, Shapiro-Wilk test, and skewness and kurtosis were calculated.

Serum fibulin-1 levels were not normally distributed ($n=168$; $p<0.0001$). For multivariate linear analyses, serum fibulin-1 levels were transformed to the natural log value to obtain a normal distribution. Between-group differences were assessed by

means of one-way analysis of covariance (ANCOVA) with adjustments for confounders with post-hoc analysis by Tukey's test.

Receiver-operator curves (ROC) were used to model the utility of serum fibulin-1 as a marker of progression. Cox regression, with and without baseline parameters, were used to model the impact of serum fibulin-1 levels on predicting progression. Kaplan-Meier survival curves were used to model the progression-free survival rate of serum fibulin-1 and between group rates were compared using the Mantel-Cox log rank test.

Parenchyma fibulin-1 levels were normally distributed (n=25; p=0.504). Between-group differences were assessed by Student's unpaired t-test.

To study the relationship between serum fibulin-1 levels and lung function parameters, correlations between serum fibulin-1 and lung function variables were analysed using Spearman's rank correlation co-efficient. To study the relationship between parenchyma fibulin-1 levels and lung function parameters, correlations between tissue fibulin-1 and lung function were analysed using Pearson's product-moment tests.

The sample size for analysis of fibroblast fibulin-1 levels was too small to determine normality (n=15). Between-group differences were assessed by means of Student's unpaired t-test.

## 2.3 Results

Baseline demographics for our study population are summarized in Table 2.4.

**Table 2.4 Baseline demographics for the study population**

| | Non-diseased control | | Sarcoidosis | | Hypersensitivity Pneumonitis | | "Other" ILD* | | Idiopathic pulmonary fibrosis | | P value ** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | n=17 | | n=12 | | n=32 | | n=35 | | n=72 | | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | |
| Age, yr | 34 | 12 | 48 | 11 | 61 | 11 | 59 | 14 | 68 | 9 | <0.0001 |
| BMI, kg/m2 | n/a | n/a | 31 | 5 | 31 | 5 | 26 | 5 | 30 | 6 | ns |
| Baseline $FEV_1$, % predicted | n/a | n/a | 79 | 23 | 70 | 19 | 74 | 22 | 79 | 20 | ns |
| Baseline FVC, % predicted | n/a | n/a | 91 | 22 | 68 | 22 | 78 | 24 | 74 | 20 | ns |
| Baseline $DL_{CO}$, % predicted | n/a | n/a | 70 | 23 | 49 | 15 | 48 | 20 | 41 | 16 | ns |
| Baseline CPI, units | n/a | n/a | 22 | 19 | 46 | 14 | 44 | 18 | 52 | 13 | ns |
| Baseline TLC, % predicted | n/a | n/a | 88 | 20 | 73 | 20 | 76 | 22 | 67 | 13 | ns |
| | | | | | | | | | | | |
| | Count | % | Count | % | Count | % | Count | % | Count | % | P value *** |
| Male | 5 | 29 | 7 | 58 | 10 | 31 | 10 | 29 | 41 | 57 | <0.0001 |
| History of smoking | 1 | 6 | 2 | 17 | 16 | 50 | 14 | 40 | 45 | 63 | <0.0001 |

Serum was collected from 151 patients with interstitial lung disease (ILD) and 17 subjects without lung disease.

*"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).
** P value for unpaired t-test, non-diseased control vs. all ILDs
***P value for Chi-squared test, non-diseased control vs. all ILDs

BMI body mass index, $FEV_1$ forced expiratory volume in 1 second, FVC forced vital capacity, $DL_{CO}$ diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity, Yr year, n/a Not available, ns Not significant

### 2.3.1 Serum fibulin-1 is increased in patients with IPF compared to other ILDs and subjects without lung disease

The level of serum fibulin-1 was greatest in IPF compared to the other ILDs after adjustments for potential confounding variables – age, gender and smoking history (Figure 2.6). Levels of serum fibulin-1 were greater in patients with IPF compared to subjects without lung disease.

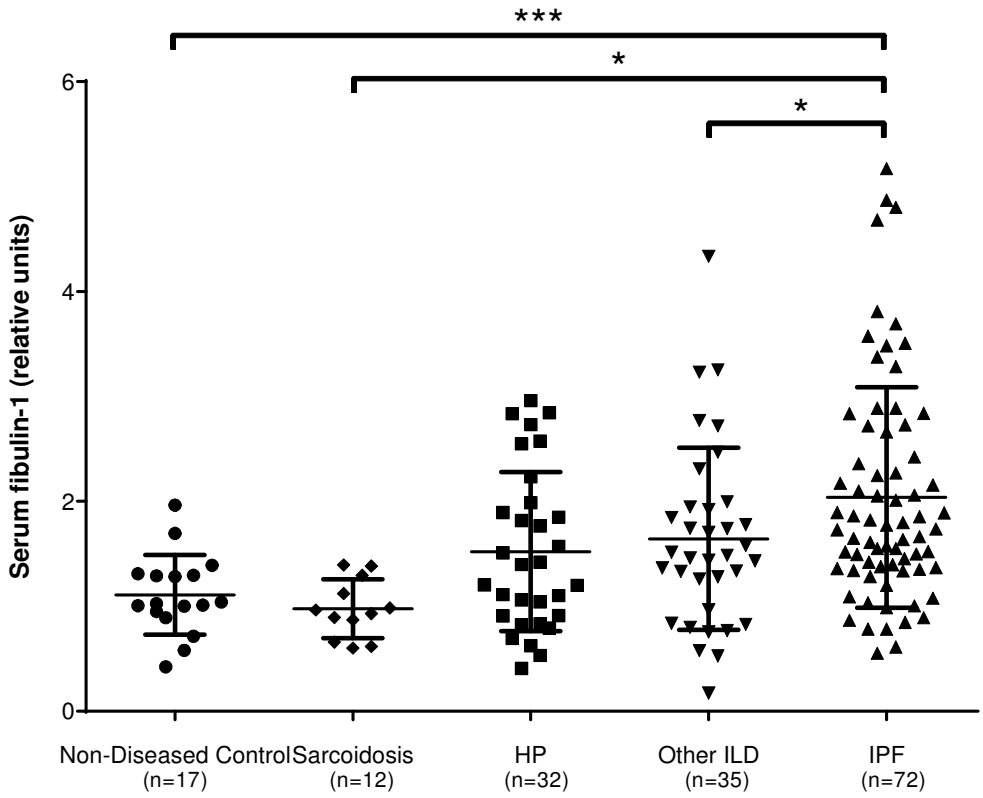



**Figure 2.6 Serum fibulin-1 levels are increased in patients with IPF compared to non-diseased controls.**
The fibulin-1 level of each serum sample was determined by immunoblotting and reported as relative to a standard serum sample. Densitometric analysis was performed using a Kodak Image Station 4000MM (Carestream Molecular Imaging Software v. 5.3.3.17476). Serum fibulin-1 levels were adjusted for age, gender and smoking history. (Analysis of covariance, n=168, post-test Tukey's *p<0.05. ***p=0.006, median ± 25th & 75th percentiles).
"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).
HP hypersensitivity pneumonitis, ILD interstitial lung disease, IPF idiopathic pulmonary fibrosis

### 2.3.2 Serum fibulin-1 correlates with disease severity in patients with ILDs

Serum fibulin-1 correlates with CPI in patients with ILDs. The CPI is a representation of the morphological extent of fibrosis as seen on high resolution computed tomography (Wells et al. 2003). Spearman's rank correlation analysis was used to model the relationship between serum fibulin-1 and CPI in patients with ILDs. (rho=0.34, p=0.01, n=73) (Figure 2.7).

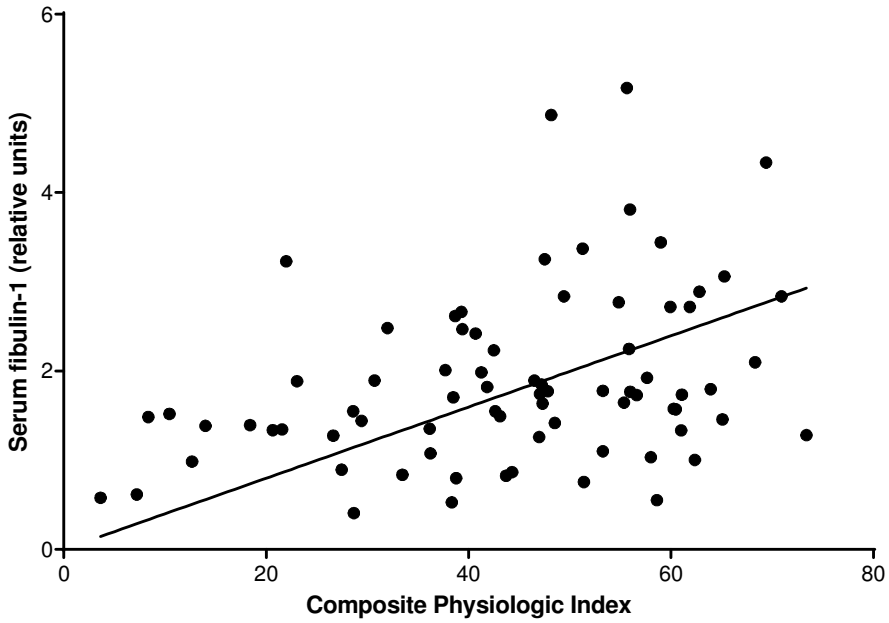



**Figure 2.7 Serum fibulin-1 levels correlate with disease severity in patients with fibrotic ILD**
The composite physiologic index (CPI) was calculated in 73 patients with interstitial lung disease (ILD). CPI was derived from lung function measurements and provided a representative measurement of disease severity.
The fibulin-1 level of each serum sample was determined by immunoblotting and reported as relative to a standard serum sample. Densitometric analysis was performed using Kodak Image Station 4000MM (Carestream Molecular Imaging Software v. 5.3.3.17476). The linear regression line was calculated by least squares methodology and the significance of the relationship was determined using Spearman's rank correlation analysis (rho=0.34, p=0.01).

Lung function measurements were used to assess disease severity in patients with ILDs. Serum fibulin-1 correlated with FVC% (rho =-0.3, p=0.01), $DL_{CO}$% (rho=-0.25, p=0.04), CPI (rho =0.34, p=0.01) (Figure 2.7) and TLC% (rho =-0.34, p=0.01) but not with $FEV_1$% (Spearman's rho correlation, Table 2.5).

Neither age, BMI, nor smoking status correlated with serum fibulin-1 levels.

**Table 2.5 Serum fibulin-1 correlates with lung function parameters in patients with ILDs**

| Variable | Spearman's correlation coefficient | p value |
|---|---|---|
| Age, yr | 0.17 | 0.20 |
| BMI, kg/m2 | 0.10 | 0.49 |
| Baseline $FEV_1$, % predicted | -0.14 | 0.23 |
| Baseline FVC, % predicted | -0.30 | 0.01 |
| Baseline $DL_{CO}$, % predicted | -0.25 | 0.04 |
| Baseline CPI, units | 0.34 | 0.01 |
| Baseline TLC, % predicted | -0.34 | 0.01 |
| | Pearson chi-square | p value |
| Male | 0.001 | 0.98 |
| History of smoking | 0.40 | 0.82 |

Lung function parameters and demographic information was obtained for 73 patients with interstitial lung disease (ILD). The time between serum collection and lung function measurements was no more than 15 days (±5). Continuous variables were analysed with spearman's rank correlation analysis and categorical variables were analysed by Pearson chi-square test. Serum fibulin-1 was measured by immunoblot. BMI body mass index, $FEV_1$ forced expiratory volume in 1 second, FVC forced vital capacity, $DL_{CO}$ diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity, Yr year

### 2.3.3 The level of fibulin-1 in whole lung lysates was increased in patients with IPF compared to subjects without lung disease

The presence of fibulin-1 in the tissue of patients with IPF was then examined specifically. The level of tissue fibulin-1 in whole lung lysates from patients with IPF (n=4, mean 3.43 units, SD 0.91) was 2.6 fold higher than in non-diseased controls (n=4, mean 1.34, SD 0.2; p=0.02) (Figure 2.8).

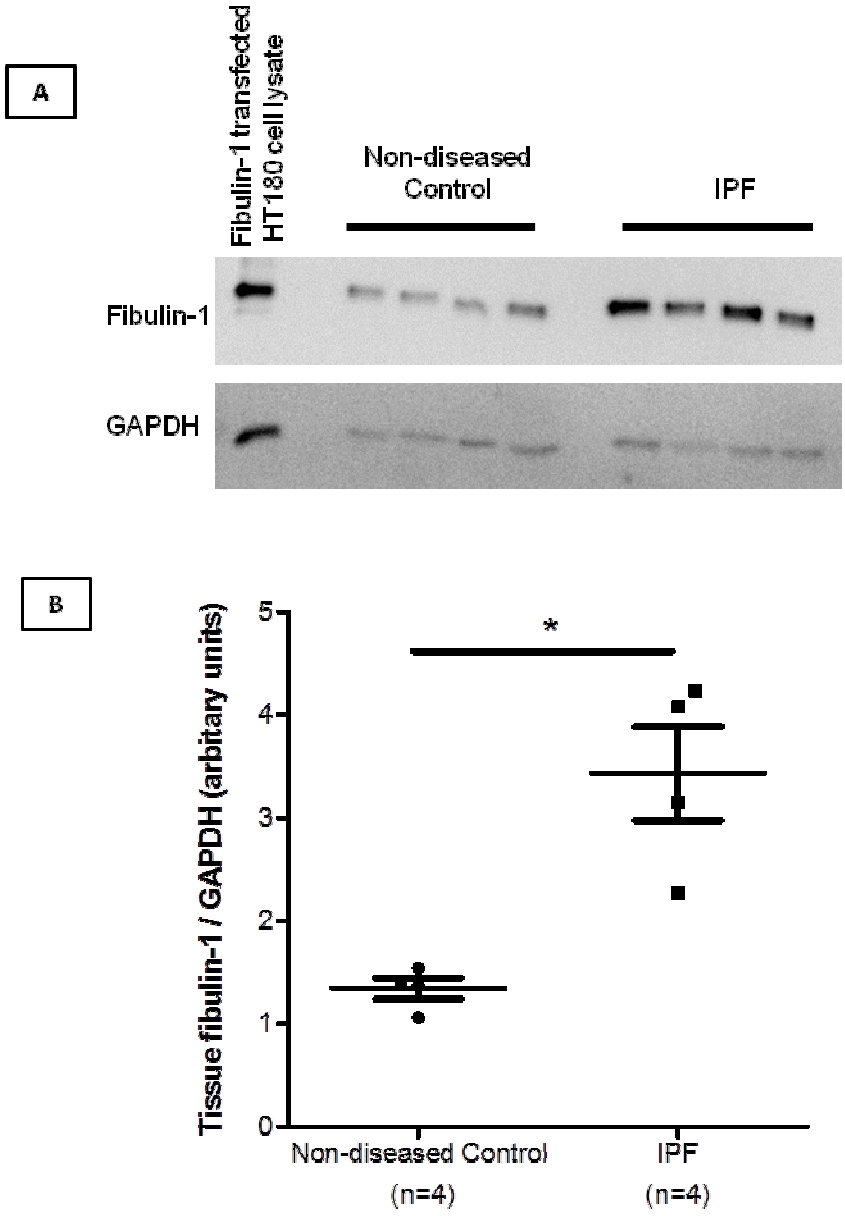



**Figure 2.8 Tissue fibulin-1 is increased in the whole lung lysate derived from patients with IPF compared to subjects without lung disease**
(A) The level of fibulin-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in whole lung lysates derived from patients with and without IPF was determined by immunoblot. The cell lysate of the fibulin-1 transfected cell line (HT-1080) was used as a positive control. Densitometric analysis was performed using Kodak Image Station 4000MM (Carestream Molecular Imaging Software v. 5.3.3.17476). The intensity of the fibulin-1 band was normalised to the intensity of the GAPDH band and reported in arbitrary units.
(B) The relative levels of fibulin-1 of subjects without disease and patients with IPF were compared using unpaired t-test. Values are expressed as means ± SD, *p=0.02.

### 2.3.4 Tissues from patients with IPF show greater levels of fibrosis.

As a positive control ensuring the presence of fibrosis in our samples, we measured the levels of total collagen in tissues from patients with IPF. Patient information is found in Table 2.6.

More total collagen (percentage area stained with Masson's trichrome) was measured in tissue from IPF patients (n=12, mean 25.0% area, SD 10.5) than in tissue from subjects without lung disease (n=5, mean 12.1, SD 6.4; p=0.02) (Figure 2.9).

**Table 2.6 Characteristics of subjects used for lung tissue analysis**

| | Non-Diseased Control (n=5) | | Idiopathic pulmonary fibrosis (n=20) | | p value* |
|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | |
| Age, yr | 38 | 14 | 59 | 8 | 0.03 |
| $FEV_1$, % predicted | n/a | n/a | 77 | 21 | n/a |
| FVC, % predicted | n/a | n/a | 74 | 20 | n/a |
| DLco, % predicted | n/a | n/a | 42 | 20 | n/a |
| CPI, units | n/a | n/a | 52 | 16 | n/a |
| TLC, % predicted | n/a | n/a | 81 | 18 | n/a |
| | | | | | |
| | Number | % | Number | % | p value** |
| Male | 4 | 80 | 14 | 70 | 0.24 |

*P value for unpaired t-test
**P value for Fisher's exact test
Continuous data were compared by use of unpaired t-tests, and categorical data by Fisher's exact tests
$FEV_1$ forced expiratory volume in 1 second, FVC forced vital capacity, $DL_{CO}$ diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity, Yr year

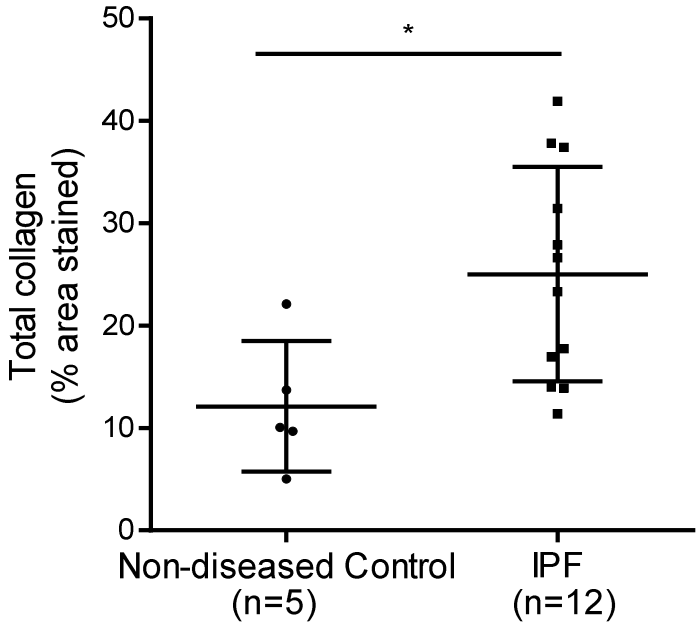



**Figure 2.9 Tissues from patients with IPF are more fibrotic compared to tissues from subjects without lung disease.**
Paraffin-embedded formalin fixed tissue sections from subjects without lung disease (n=5) and patients with IPF (n=12) were stained by Masson's trichrome. Levels of total collagen were quantified by computer aided image analysis using ImageJ and reported as the percentage area of tissue components stained green. Twenty images from each tissue section were analysed and 2-6 tissue sections were measured for each subject. Averaged staining levels of collagen between the groups were compared using unpaired t-test. (*$p<0.05$). Values are expressed as mean ± SD.
IPF idiopathic pulmonary fibrosis, SD standard deviation

### 2.3.5 The level of fibulin-1 in paraffin embedded formalin fixed tissue was increased in patients with IPF compared to subjects without lung disease

In patients with IPF, the level of tissue fibulin-1 detected in paraffin embedded formalin fixed tissue sections was significantly higher (n=20, mean 129.2 units, SD 4.32) compared to non-diseased controls (n=5, mean 118.6, SD 3.73) ($p<0.001$). Age was not a confounding factor. There was no significant difference in the level of tissue fibulin-1 between samples taken from patients with IPF whether the tissue originated from a diagnostic surgical lung biopsy or from explanted lung post-transplant ($p=0.24$)(Figure 2.10).

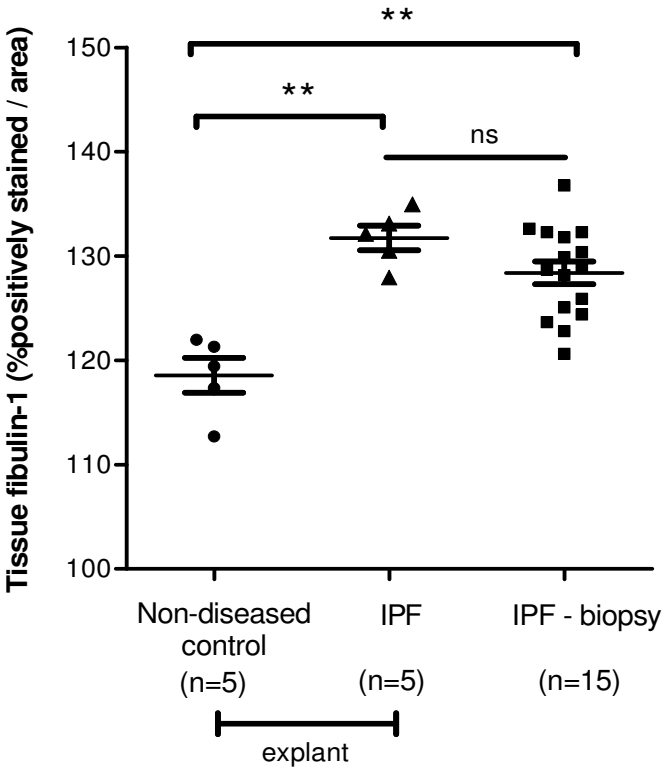



**Figure 2.10 Tissue sections derived from patients with IPF have greater fibulin-1 levels compared to sections derived from subjects without lung disease.**

Paraffin-embedded formalin fixed tissue sections were stained for fibulin-1 and counterstained for all other tissue components. Levels of fibulin-1 were quantified by computer aided image analysis using ImageJ and reported as the area positively stained for fibulin-1 as a percentage of the total area stained. Twenty images of each tissue section were analysed, 2-6 tissue sections were measured for each subject. Subsequently, measurements of each image were averaged together to obtain a single value per subject/patient.
Levels of fibulin-1 compared using one-way analysis of variance and Tukey's multiple comparison post-test (**$p<0.005$).
Values are expressed as mean ± SD.
IPF idiopathic pulmonary fibrosis, SD standard deviation
Details on staining quantification can be found in 2.2.14 and seen in Figure 2.5

### 2.3.6 Tissue fibulin-1 levels correlate with disease severity in patients with IPF

Higher tissue fibulin-1 levels correlated significantly with lower $FEV_1$% and FVC% measurements (FEV%1 r=-0.86, p=0.014, FVC% r=-0.92, p=0.004) (Figure 2.11). Tissue fibulin-1 levels did not significantly correlate with age, $DL_{CO}$%, CPI, or TLC% (Table 2.7). All samples for correlation analysis were from Modena and the time between lung function test and biopsy was not more than 30 (±5) days.

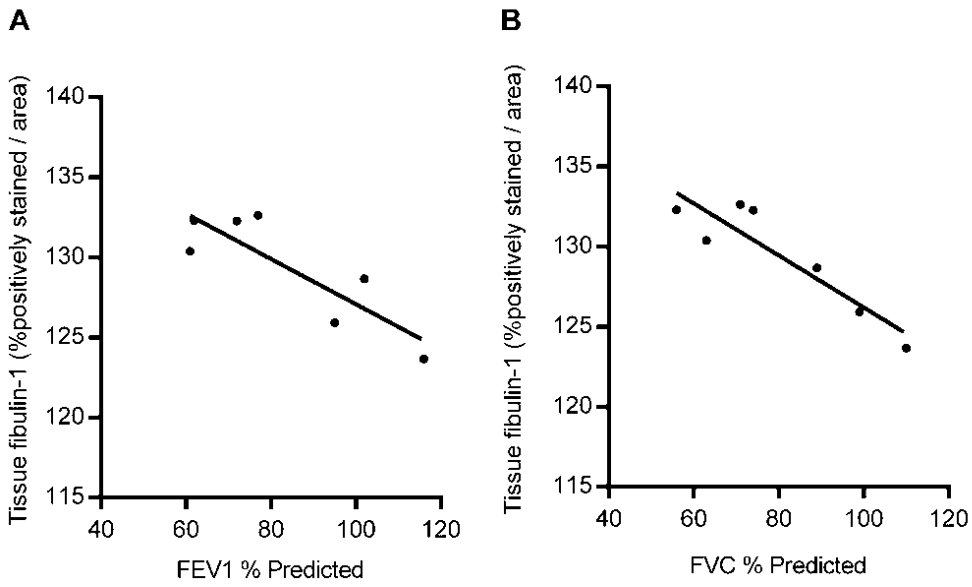



**Figure 2.11 . Lung tissue fibulin-1 levels in patients with IPF inversely correlate with lung function measurements.**
Paraffin-embedded formalin fixed tissue sections were stained for fibulin-1 and counterstained for all other tissue components. Levels of fibulin-1 were quantified by computer aided image analysis using ImageJ and reported as the area positively stained for fibulin-1 as a percentage of the total area stained. Twenty images of each tissue section were analysed and 2-6 tissue sections were measured for each subject. Subsequently, measurements of each image were averaged to obtain a single value per patient. Averaged tissue fibulin-1 levels for each patient (n=7) was compared to their (A) $FEV_1$ and (B) FVC percentage predicted measurements. The linear regression line represents Pearson product-moment coefficients ($FEV_1$ r=-0.86, p=0.014, FVC r=0.92, p=0.004). The time between biopsy and lung function measurements was 30 days (±5).

$FEV_1$ forced expiratory volume in 1 second, FVC forced vital capacity, IPF idiopathic pulmonary fibrosis

**Table 2.7 Tissue fibulin-1 correlates with percentage predicted $FEV_1$ and FVC measurements in patients with idiopathic pulmonary fibrosis**

| Variable | Pearson Correlation Coefficient | P-value |
|---|---|---|
| $FEV_1$, % predicted | -0.855 | 0.014 |
| FVC, % predicted | -0.916 | 0.004 |
| $DL_{CO}$, % predicted | -0.589 | 0.164 |
| TLC, % predicted | -0.786 | 0.064 |
| CPI | 0.665 | 0.103 |
| Age, yr | -0.624 | 0.135 |

Levels of fibulin-1 were quantified by computer aided image analysis using ImageJ and reported as the area positively stained for fibulin-1 as a percentage of the total area stained. Twenty images of each tissue section were analysed, 2-6 tissue sections were measured for each subject. Subsequently, measurements of each image were averaged to obtain a single value per patient. Two to six surgical lung biopsies and lung function parameters were obtained from each of 7 patients with idiopathic pulmonary fibrosis. The relationship between tissue fibulin-1 level and lung function variables were examined by Pearson correlation analysis. The time between biopsy and lung function measurements was 30 days (±5).

$FEV_1$ forced expiratory volume in 1 second, FVC forced vital capacity, $DL_{CO}$ diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity, Yr year

### 2.3.7 Fibroblasts from IPF patients produce more fibulin-1 mRNA under basal conditions than fibroblasts from subjects without lung disease

To investigate the role of the fibroblast in the production of fibulin-1, fibroblasts derived from the distal parenchyma of patients with and without IPF were compared. Initially, the levels of fibulin-1 at the transcription level were examined.

Fibroblasts isolated from patients with IPF and from age and gender matched subjects without IPF were cultured under non-stimulatory conditions. Patient information from whom the fibroblasts were isolated are found in Table 2.8.

Equal amounts of mRNA from each fibroblast line were converted into cDNA and levels of mRNA were quantified by QPCR. The comparative (or ΔΔCt) method was used to calculate the relative abundance of mRNA compared to the housekeeping gene 18S. Samples with a high amount of a particular mRNA transcript have a smaller delta Ct value because fewer cycles of PCR are required to amplify the target gene to a pre-determined threshold value. Fibroblasts from patients with IPF (n=5, mean 9.05 cycles, SD 0.70) produced more fibulin-1 mRNA under basal conditions compared to fibroblasts from subjects without IPF (n=4, mean 11.17, SD 0.40, p=0.03) (Figure 2.12).

Therefore, for ease of reference, mRNA levels are displayed as 1/delta Ct.

**Table 2.8 Patient information from whom fibroblasts were derived**

| Donor # | Gender | Age (Yrs) | Diagnosis | Surgery |
|---|---|---|---|---|
| Non-IPF 1 | Male | 55 | NSCLC | Resection |
| Non-IPF 2 | Male | 66 | NSCLC | Resection |
| Non-IPF 3 | Female | 54 | NSCLC | Resection |
| Non-IPF 4 | Male | 60 | NSCLC | Resection |
| Non-IPF 5 | Male | 57 | NSCLC | Resection |
| Non-IPF 6 | Male | 59 | NSCLC | Resection |
| Non-IPF 7 | Male | 61 | NSCLC | Resection |
| IPF 1 | Male | 53 | Idiopathic pulmonary fibrosis | Transplant |
| IPF 2 | Male | 62 | Idiopathic pulmonary fibrosis | Transplant |
| IPF 3 | Male | 57 | Idiopathic pulmonary fibrosis | Transplant |
| IPF 4 | Male | 55 | Idiopathic pulmonary fibrosis | Transplant |
| IPF 5 | Male | 58 | Idiopathic pulmonary fibrosis | Transplant |
| IPF 6 | Male | 58 | Idiopathic pulmonary fibrosis | Transplant |
| IPF 7 | Male | 54 | Idiopathic pulmonary fibrosis | Transplant |
| IPF 8 | Male | 63 | Idiopathic pulmonary fibrosis | Transplant |

Parenchymal fibroblasts were isolated from lung tissue obtained from donors undergoing thoracotomy for either resection or transplantation. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma (NSCLC). Pulmonary function and smoking data were not available for these patients.
IPF idiopathic pulmonary fibrosis, Yrs years

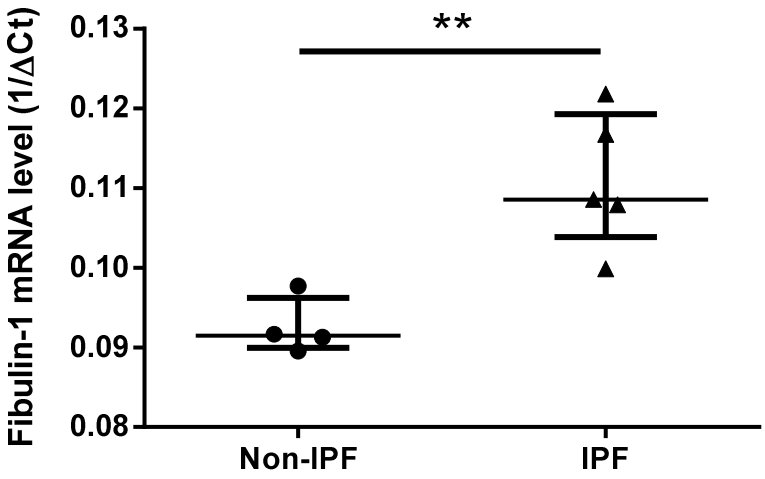



**Figure 2.12 Fibroblasts derived from patients with IPF produce more fibulin-1 mRNA under basal conditions than fibroblasts from patients without IPF.**

Primary parenchymal fibroblasts from patients with IPF (n=8) and age and gender matched patients without IPF (n=7) were grown for 72 hours in 5% FBS/ DMEM/ 1% Pen-Strep, quiesced in 0.1% FBS/ DMEM/ 1% pen-strep for 24hours and maintained in fresh 0.1% FBS/ DMEM / 1% pen-strep for a further 72 hours. Non-IPF fibroblasts are derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. 500ng of mRNA was converted to cDNA for this comparison. **p=0.003 unpaired t-test Values are expressed as median and interquartile range.
Data are expressed as 1/delta cycle threshold to 18S (ΔCt) to enable a greater number to reflect more mRNA.

IPF idiopathic pulmonary fibrosis, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

### 2.3.8 Fibroblasts from IPF patients produce more fibulin-1 than fibroblasts from non-IPF fibroblasts

The production of the two forms of fibulin-1 protein, secreted and cell-associated were examined. The secreted and cell-associated levels of fibulin-1 were compared between primary parenchymal fibroblasts from patients with IPF and with age and gender matched subjects without IPF under non-stimulated cell culture conditions and in fibroblasts at low passage number ($\leq$ 5 passages). Cell-associated levels include intracellular, extracellular, and cell surface-bound proteins.

Fibroblasts derived from patients with IPF produced significantly more secreted (measured by cell culture supernatant) fibulin-1 than fibroblasts derived from subjects without lung disease ($p=0.003$) (Figure 2.13A). In addition, fibroblasts from patients with IPF produced more cell-associated (measured by both intra- and extra-cellular protein) fibulin-1 than fibroblasts derived from subjects without lung disease ($p=0.007$) (Figure 2.13B).

The stability of the level of fibulin-1 between cell passages is not known and could be investigated in future studies. In these studies, cell cultures at less than 6 passages were used.

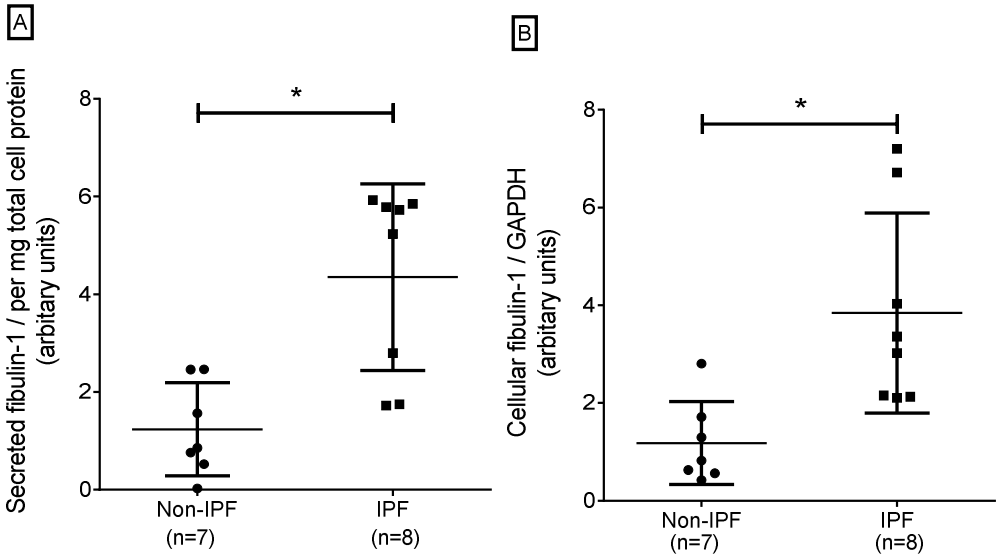



**Figure 2.13 Parenchymal fibroblasts from patients with IPF produce more fibulin-1 than fibroblasts from patients without IPF.**

Primary parenchymal fibroblasts from patients with IPF (n=8) and age and gender matched patients without IPF (n=7) were grown for 72hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24hours and maintained in fresh 0.1% FBS/DMEM/1% pen-strep for a further 72hours. Supernatants and cell lysates were collected and analysed by western blot. Densitometric values were normalised to total protein of the cell monolayer (supernatants, A) or GAPDH detected on the same blots (cell lysates, B). Unpaired t-test **$p<0.01$. Values are expressed as median and interquartile range.
Non-IPF fibroblasts are derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. IPF idiopathic pulmonary fibrosis, GAPDH Glyceraldehyde 3-phosphate dehydrogenase, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

### 2.3.9 Serum levels of fibulin-1 between patients with IPF were similar across patient cohorts

There were no significant differences in fibulin-1 levels between patients with IPF from the 3 cohorts used in this study. Patients with IPF from San Francisco had the poorest lung function of the groups (Table 2.9).

**Table 2.9 Patient characteristics of the patients with IPF from 3 independent populations.**

| | Cohort 1 | | Cohort 2 | | Cohort 3 | | | |
|---|---|---|---|---|---|---|---|---|
| Variable | Sydney IPF (n=27) | | Modena IPF (n=28) | | San Francisco IPF (n=17) | | *ANOVA | **Tukey's post-hoc (between group, p value) |
| | Mean | SD | Mean | SD | Mean | SD | p value | |
| Age, yr | 67.7 | 7.7 | 65.9 | 10.1 | 71.6 | 9.7 | 0.225 | n/a |
| BMI, kg/m2 | 30.5 | 6.4 | n/a | n/a | n/a | n/a | n/a | n/a |
| Baseline $FEV_1$%, % predicted | 82.7 | 20.5 | 80.6 | 22.0 | 72.3 | 14.0 | 0.248 | n/a |
| Baseline FVC%, % predicted | 78.2 | 19.9 | 77.7 | 21.6 | 62.4 | 14.3 | 0.021 | (1 vs 3, 0.03)<br>(2 vs 3, 0.04) |
| Baseline $DL_{CO}$%, % predicted | 41.0 | 16.2 | 42.3 | 18.0 | 38.9 | 15.6 | 0.838 | n/a |
| Baseline CPI, units | 51.0 | 12.2 | 49.0 | 14.5 | 57.8 | 10.0 | 0.129 | n/a |
| Baseline TLC%, % predicted | 66.8 | 11.2 | 75.6 | 12.1 | 62.0 | 13.0 | 0.039 | (2 vs 3, 0.03) |
| First blood draw serum fibulin-1, units | 2.0 | 0.7 | 2.0 | 1.1 | 2.2 | 1.4 | 0.800 | n/a |
| | | | | | | | | |
| | Number | % | Number | % | Number | % | | |
| Male | 21 | 77.80 | 10 | 35.70 | 10 | 83.30 | 0.00 | (1 vs 2, 0.002)<br>(2 vs 3, 0.008) |
| History of smoking | 17 | 63.00 | 17 | 60.70 | 11 | 70.60 | 0.21 | n/a |

*One way analysis of variance (ANOVA)

**Significant ANOVA comparisons were then analysed *post-hoc* for between group differences using Tukey's

IPF idiopathic pulmonary fibrosis, SD standard deviation, n/a not applicable, yr years, $FEV_1$ forced expiratory volume in 1 second, FVC forced vital capacity, $DL_{CO}$ diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity

Patients with IPF, combined from the 3 independent populations, had significantly higher serum fibulin-1 levels than subjects without lung disease (Figure 2.14).

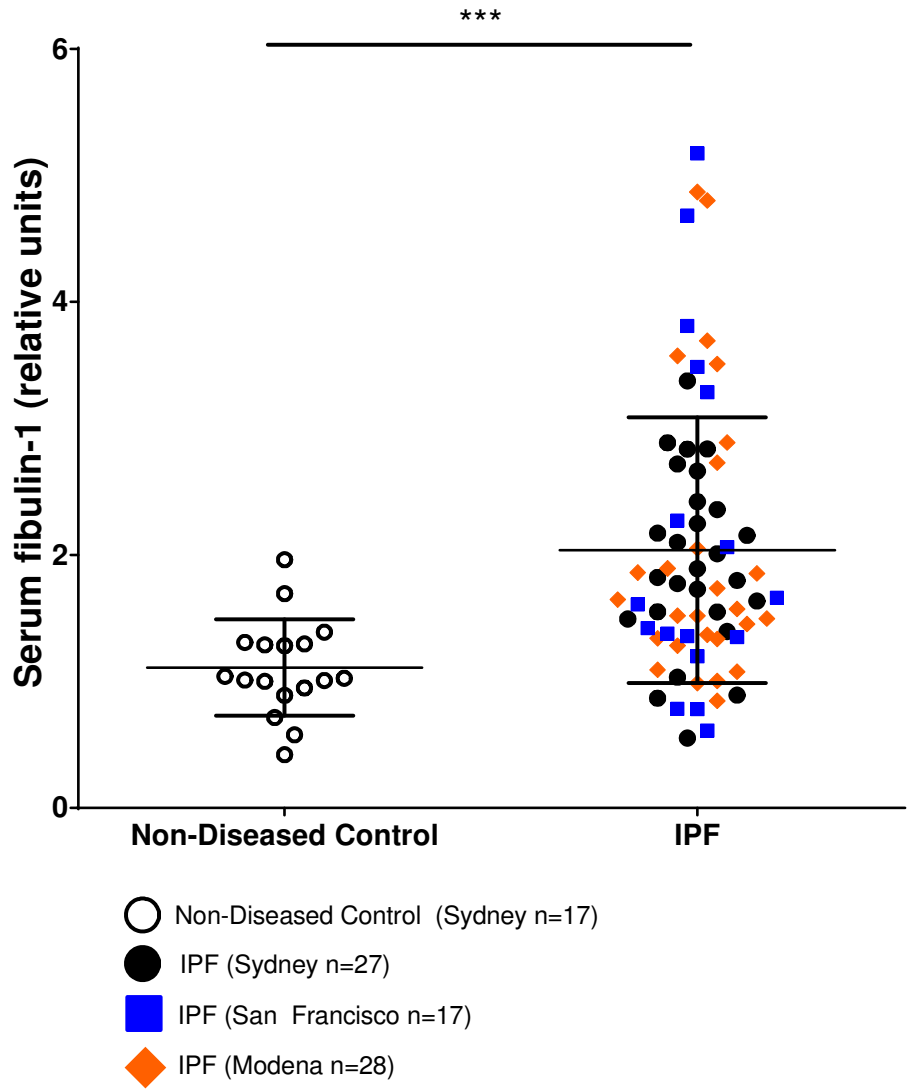



**Figure 2.14 Patients with IPF had significantly higher levels of serum fibulin-1 compared to subjects without lung disease.**
Serum fibulin-1 levels in patients with IPF, measured by western blot analysis of equal volumes of serum, were normalised against a standard serum sample loaded onto every gel. Densitometric values were transformed to natural log values before analysis. Data were adjusted for age, gender and smoking history. (ANCOVA, Sydney cohort n=27, black circle, San Francisco cohort n=17, black square, Modena cohort n=28, grey diamond, post-test Tukey's ***p=0.006, median ± 25th & 75th percentiles).
IPF idiopathic pulmonary fibrosis, ANCOVA analysis of covariance

### 2.3.10 Serum fibulin-1 was increased in patients with IPF who progressed compared to those who remained stable

The utility of serum fibulin-1 as a biomarker of disease progression in patients with IPF was then investigated. There were a total of 72 patients with IPF and of those 48 had follow up information available. Patient characteristics of the patients with IPF used in the progression analysis are found in Table 2.10.

**Table 2.10 Characteristics of patients with IPF used in progression analysis**

| | **All IPF** | | **Stable** | | **Progressed** | | **p value *** |
|---|---|---|---|---|---|---|---|
| | n=72 | | n=21 | | n=27 | | |
| | Mean | SD | Mean | SD | Mean | SD | |
| Age, yr | 68 | 9 | 65 | 11 | 70 | 9 | ns |
| $FEV_1$, % predicted | 79 | 20 | 79 | 19 | 76 | 20 | ns |
| FVC, % predicted | 74 | 20 | 78 | 18 | 68 | 21 | ns |
| $DL_{co}$, % predicted | 41 | 16 | 48 | 18 | 33 | 14 | 0.012 |
| CPI, units | 52 | 13 | 44 | 14 | 58 | 10 | 0.002 |
| TLC, % predicted | 67 | 13 | 75 | 12 | 63 | 12 | 0.027 |
| Serum fibulin-1, units | 2.04 | 1.05 | 1.59 | 0.83 | 2.34 | 1.18 | 0.013 |
| | | | | | | | p value ** |
| | Number | % | Number | % | Number | % | |
| Male | 41 | 57 | 10 | 48 | 15 | 56 | ns |
| History of smoking | 45 | 63 | 14 | 74 | 19 | 70 | ns |

*P value for Kruskal-Wallis test
**P value for Pearson Chi-squared test

Of the total of 72 patients with IPF, 48 had follow up information available. Continuous data of patients with IPF that either remained stable or progressed in the year following blood draw were compared by the Kruskal-Wallis test, and categorical data were compared with Pearson's chi-squared test.
IPF idiopathic pulmonary fibrosis, SD standard deviation, yr years, $FEV_1$ forced expiratory volume in 1 second, FVC forced vital capacity, $DL_{co}$ diffusing capacity of carbon monoxide, CPI composite physiologic index, TLC total lung capacity, ns non significant

Patients with IPF who later experienced a progression event had a higher level of serum fibulin-1 at the time of blood draw compared to patients with IPF who remained stable (Table 2.10). As expected, and as supported by evidence in the literature, those who progressed also had a poorer $DL_{CO}$%, CPI and TLC% than those that remained stable (Best et al. 2008).

### 2.3.11 Serum fibulin-1 predicts disease progression in patients with IPF

To investigate the ability of serum fibulin-1 levels to discriminate between patients who progressed and those who remained stable, receiver-operating characteristic (ROC) curves were constructed.

The area under the ROC curve was 0.71 (95%CI 0.57-0.86, p=0.012) (Figure 2.15A). Kaplan-Meier curves showed that IPF patients with a serum fibulin-1 level > 1.6 units had a shorter progression-free survival time than those with ≤ 1.6 units (p=0.003) (Figure 2.15B)

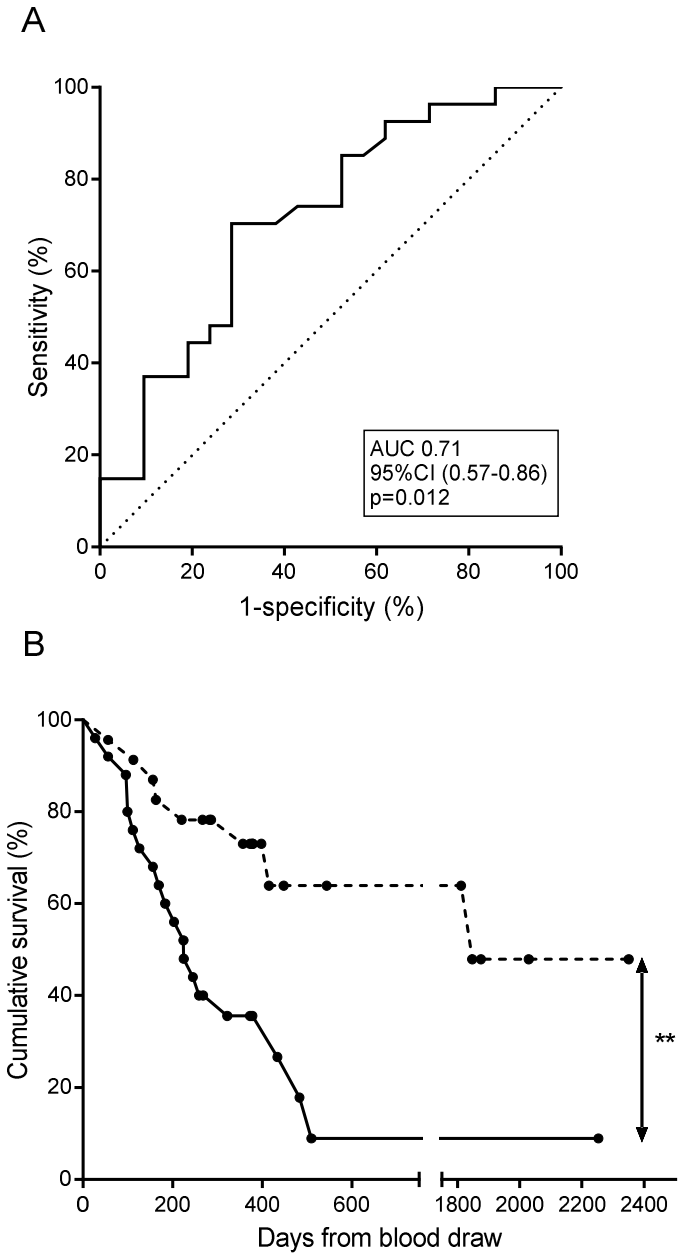



**Figure 2.15 Serum fibulin-1 level predicts disease progression patients with IPF.** Patients with IPF were followed up for at least one year (365 ± 1 day) following blood draw. (A) Fibulin-1 levels in patients who progressed (n=27) were compared to the levels in patients who did not progress (n=21) and a receiver operating characteristic (ROC) curve was generated. (B) Kaplan-Meier survival curves showing time to progression event were generated. Patients who had a serum fibulin-1 of less than 1.6 units (n=23, dashed line) were compared to patients who had a level of more than 1.6 units (n=25, solid line). Circles on each line represent time censoring. Mantel-Cox log rank **p=0.003.

AUC area under the curve, CI confidence interval, IPF idiopathic pulmonary fibrosis

From the ROC, thresholds of serum fibulin-1 were used to estimate the ability of serum fibulin-1 to predict disease progression. The sensitivities and specificities of the test are found in Table 2.9.

Three threshold values differing in their respective sensitivities and specificities were chosen for further analysis. The three thresholds are ≤ 1.1 units, ≤1.6 units and ≤2.9 units.

At a threshold of 1.6 units, serum fibulin-1 identified patients with IPF who progressed with 70% sensitivity and 71% specificity. At thresholds of 1.1 and 2.9 units, the sensitivities and specificities were 93% and 33%, and 30% and 90% respectively (Table 2.11).

**Table 2.11 Validity of serum fibulin-1 thresholds in patients with IPF to predict disease progression**

| Representative threshold value | Sensitivity | Specificity | Used for further analysis |
|---|---|---|---|
| 0.697 | 100% | 5% | |
| 0.815 | 100% | 14% | |
| 1.085 | 93% | 33% | * |
| 1.309 | 89% | 38% | |
| 1.355 | 85% | 48% | |
| 1.417 | 74% | 48% | |
| 1.535 | 70% | 62% | |
| 1.592 | 70% | 71% | * |
| 1.810 | 52% | 71% | |
| 1.857 | 48% | 76% | |
| 2.076 | 41% | 81% | |
| 2.500 | 37% | 86% | |
| 2.888 | 30% | 90% | * |
| 3.088 | 26% | 90% | |
| 3.497 | 15% | 90% | |

Selected threshold values from the ROC curve (Area under the curve = 0.71, 95% confidence interval 0.57 – 0.86, p=0.012, n=48). Values with * were used for further analysis

IPF, idiopathic pulmonary fibrosis

### 2.3.12 Patients with IPF and a high level of serum fibulin-1 had a shorter progression-free survival time than those with a low level of serum fibulin-1

Additional threshold levels were also analysed for their ability to predict disease progression in patients with IPF. At three thresholds of serum fibulin-1, those that had a higher level of serum fibulin-1 had a shorter progression-free survival time compared to those that had a lower level of serum fibulin-1 at the time of blood draw. The case processing summary for these comparisons is found in Table 2.12.

The progression-free survival rates of patients with IPF with high or low serum fibulin-1 levels at the time of blood draw were also significantly different if thresholds of 1.1 units or 2.9 units were used to stratify the groups (1.1 units p=0.016, 2.9 units p=0.019) (Table 2.12).

**Table 2.12 Patients with IPF who presented with a high level of serum fibulin-1 had a shorter progression free survival time compared to those who had a low level of serum fibulin-1**

| | | | Average time to progression event (days) | | | | |
|---|---|---|---|---|---|---|---|
| **Threshold 1.1 units** | Total N | N of Progressed | Estimate | Std. Error | 95% CI | | p value |
| | | | | | Lower | Upper | |
| Low fibulin-1 | 10 | 2 | 1772 | 360 | 1066 | 2478 | 0.016 |
| High fibulin-1 | 38 | 25 | 704 | 167 | 377 | 1031 | |
| **Threshold 1.6 units** | Total N | N of Progressed | Estimate | Std. Error | 95% CI | | p value |
| | | | | | Lower | Upper | |
| Low fibulin-1 | 23 | 8 | 1509 | 241 | 1036 | 1982 | 0.003 |
| High fibulin-1 | 25 | 19 | 433 | 156 | 128 | 738 | |
| **Threshold 2.9 units** | Total N | N of Progressed | Estimate | Std. Error | 95% CI | | p value |
| | | | | | Lower | Upper | |
| Low fibulin-1 | 39 | 20 | 1056 | 197 | 670 | 1441 | 0.019 |
| High fibulin-1 | 9 | 7 | 215 | 35 | 146 | 283 | |

The progression-free survival time of patients who presented with a high level of fibulin-1 (greater than the threshold of 1.1, 1.6 or 2.9) and low level of fibulin-1 (less than the threshold of 1.1, 1.6 or 2.9) were compared using the Mantel-Cox log rank test. N= number, IPF= idiopathic pulmonary fibrosis, CI confidence interval

### 2.3.13 Measurement of serum fibulin-1 predicts progression in patients with IPF independent of other predictors.

Due to the small number of patients with IPF available for follow up analysis, only a few traditional predictors of disease progression were included in the cox regression models. The predictors of disease progression in IPF included in the models were baseline age, history of smoking, FVC%, and $DL_{CO}$%.

An increase in one unit of serum fibulin-1 level in patients with IPF carried a significant hazard ratio for the likelihood of disease progression of 1.69 (95%CI 1.2 – 2.3, p=0.001).

Univariate logistic regression showed that age, history of smoking, or FVC% did not predict disease progression in our study population. When the variables serum fibulin-1, age, history of smoking, FVC%, and $DL_{CO}$% were analysed simultaneously in the multivariate model, the independent contribution of serum fibulin-1 increased to 2.11 (95%CI 1.3 – 3.5, p=0.004) (Table 2.13). $DL_{CO}$% did not predict disease progression in the multivariate model.

Finally, an IPF patient with a level of serum fibulin-1 > 1.6 units was 5 times more likely to progress compared to a patient with a level of serum fibulin-1 ≤ 1.6 units (HR 5.2, 95% CI 2.0 – 13.3, p=0.001).

**Table 2.13 Measurement of serum fibulin-1 predicts progression in patients with IPF**

| | Univariate | | | | Multivariate | | | |
|---|---|---|---|---|---|---|---|---|
| Variable | HR | 95% CI | | P value | HR | 95% CI | | P value |
| Serum fibulin-1, units | 1.691 | 1.225 | 2.335 | 0.001* | 2.109 | 1.271 | 3.500 | 0.004* |
| Age, yr | 1.019 | 0.981 | 1.058 | 0.332 | 0.965 | 0.913 | 1.019 | 0.200 |
| History of smoking | 0.939 | 0.411 | 2.149 | 0.882 | 0.879 | 0.291 | 2.649 | 0.818 |
| FVC, % predicted | 0.989 | 0.970 | 1.009 | 0.280 | 0.990 | 0.965 | 1.015 | 0.428 |
| $DL_{co}$, % predicted | 0.971 | 0.942 | 1.000 | 0.050* | 0.997 | 0.960 | 1.035 | 0.875 |

*Significant p value of <0.05
FVC forced vital capacity, $DL_{CO}$ diffusing capacity of carbon monoxide, HR hazard ratio, CI confidence interval, Yr years

Fibulin-1 was increased in all the tested materials from patients with IPF compared to subjects without lung disease or without IPF. Importantly, a potential source for the increased serum fibulin-1 was the mesenchymal fibroblast. Furthermore, levels of serum fibulin-1 were able to discriminate between IPF patients who later experienced a decline in lung function and those who did not.

Fibulin-1 may play an important role in the pathogenesis of pulmonary fibrosis and may be useful to identify patients with IPF who are at a greater risk of disease progression.

### 2.3.14 Summary of findings

Fibulin-1 is increased in materials from patients with IPF compared to individuals without IPF (Table 2.14)

**Table 2.14 Fibulin-1 is increased in patients with IPF compared to subjects without IPF**

| Sample material | *Without IPF** | *IPF* |
|---|---|---|
| Serum | ✔ | ↑ |
| Whole lung lysate | ✔ | ↑ |
| Parenchymal tissue | ✔ | ↑ |
| Primary fibroblasts | | |
| • mRNA | ✔ | ↑ |
| • Cell- secreted protein | ✔ | ↑ |
| • Cell-associated protein | ✔ | ↑ |

✓ = present/detected
↑ = increased compared to without IPF

*Primary fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. All other material was obtained from subjects without lung disease
mRNA messenger ribonucleic acid, IPF idiopathic pulmonary fibrosis

## 2.4 Discussion

In this chapter, the importance of fibulin-1 as a biomarker in IPF was examined. This chapter contained four subsections. Firstly the presence of fibulin-1 in the serum of patients with various ILDs was characterised and the level was related to that patient's lung function measurements. Secondly the presence of fibulin-1 in the tissue of patients with IPF specifically was identified and measured, and the levels were also related to the lung function measurements. Parenchymal fibroblasts from patients with IPF and age and gender matched patients without IPF were then examined as a potential source of fibulin-1. Finally, the prognostic utility of serum fibulin-1 in patients with IPF was explored.

This is the first study linking the levels of fibulin-1 with the severity and disease progression of IPF, a relentlessly progressive disease with high mortality rates. Increased levels of fibulin-1 were found in the serum, lung tissue, and primary fibroblast cultures of patients with IPF

and importantly, a high serum fibulin-1 level may serve as a biomarker for future disease progression. Moreover, the findings were derived from four separate patient cohorts from three different countries, which is important, as IPF is a variable and global disease.

Raised fibulin-1 levels in the serum and bronchoalveolar lavage fluid of patients with asthma have been previously observed by our research group (Lau et al. 2010), a disease in which fibrosis of the airways is associated with disease progression (Royce et al. 2012). Across diseases in which fibrosis occurs there may be similar mechanisms contributing to fibrogenesis (Kisseleva and Brenner 2008). Hence, it

was logical to explore whether fibulin-1 would also be important in a disease predominantly characterised by fibrosis, ie IPF. This was the case, as serum, tissue and fibroblast-produced fibulin-1 were increased in patients with IPF compared to subjects without IPF. Interestingly, while the full length of fibulin-1 was considered to be 100kDa, as this is the size of fibulin-1 isolated from placenta and used as the positive control in the western blots, serum appears to contain at least four sizes of proteins that were detected with the fibulin-1 antibody used in these studies. Within the constraints of this study, it was not possible to sequence the remaining bands to investigate whether these additional bands represented smaller fragments of fibulin-1 or other proteins.

Alterations in fibulin-1 levels have been observed in a number of diseases. Plasma levels of fibulin-1 were identified as a potential marker for kidney malfunction (Neiman et al. 2011), and increased levels of fibulin-1 were detected in sera from patients with preeclampsia (Liu et al. 2011). It is likely that the feature common to these diseases and the lung disease that was the focus of this study is active fibrosis.

Serum fibulin-1 levels were elevated across patients with various ILDs and were not specifically increased in patients with IPF. The average level of serum fibulin-1 increased as the likelihood of lung fibrosis increased within the ILDs. However, when age, gender and smoking history were taken into account, only patients with IPF have levels of serum fibulin-1 significantly greater than subjects without lung disease. Further investigation into the levels of fibulin-1 in other ILDs is warranted.

Sarcoidosis is an ILD which rarely progresses to fibrosis while IPF is invariably fibrotic. This relationship between increasing levels of serum fibulin-1 and extent of fibrosis was supported by the inverse correlations between serum fibulin-1 and lung

function among patients with ILD, although serum fibulin-1 did not correlate with lung function among patients with IPF alone. However, this could be because the study population was recruited from tertiary referral centres and there were limited numbers of IPF patients with lung function at the higher end of the scale (>70 % predicted).

In addition, in this study progression was defined as a *decline* in lung function. So if a patient had stable but poor lung function at presentation, then unless the patient died, the patient would not have been considered to have "progressed". Progression was a yes/no (discrete) event in this study. Correlation is a continuous relationship. Serum fibulin-1 doesn't perfectly identify which patients will progress (70% specificity at 1.6 units cutoff). It may be possible to overcome the limit of random chance ($p<0.05$) with a greater study population.

In correlation analysis, the strength of the relationship determines the minimum number of samples that are needed in order to correctly reject the null hypothesis (which is that there is no relationship between the two variables in question). The relationship between serum fibulin-1 at blood draw in patients with ILD and their corresponding $DL_{CO}$% value was rho=-0.25 and the number of samples tested was 73 in this study. If it is assumed that this sample relationship was true in only patients with IPF then more than 45 samples would be needed to test in order to be able to correctly reject the null hypothesis. Although our study consisted of 72 patients with IPF, only 36 of these patients had a lung function test within 15 (±5) days of blood draw and therefore could be included in the correlation analysis. Measurements of serum fibulin-1 and lung function would be needed from nine more patients with IPF in order to be able to correctly reject the null hypothesis.

If fibulin-1 were to be biologically important during active fibrosis, then levels of fibulin-1in the serum could stem from increased secretion of fibulin-1 by lung fibroblasts. The excess fibulin-1 may also be deposited into the ECM and therefore promote to disease progression by modulating cell behaviour such as increasing platelet adhesion (Godyna et al. 1995). Fibulin-1 is a provisional matrix protein (Williams and Schwarzbauer 2009) and as such is one of the first proteins to be deposited during the wound healing cascade (Midwood et al. 2004). While much of the focus in the literature has centred on the importance of collagen to fibrosis (Calabresi et al. 2007), it is possible that smaller ECM connecting proteins (Bensadoun et al. 1996), like fibulin-1 which binds to elastic fibres (Roark et al. 1995), can also act to alter the mechanical properties of the lung (Le Saux et al. 2008). In this study, as the levels of tissue fibulin-1 increased, the lung function of the patients decreased.

Fibulin-1 is a known modulator of the ECM during the progression of some diseases (Argraves et al. 2009) and therefore may be a protein to target during fibrogenesis. There is emerging evidence that the changes in ECM composition are relevant for understanding pulmonary fibrosis, as a “fibrotic” matrix induces myofibroblast differentiation (du Bois 2010). The “activated” fibroblast is central to the development of fibrogenesis (Liu et al. 2010) and our cellular studies on fibroblasts have reinforced the importance of fibulin-1 in IPF.

The primary parenchymal fibroblasts derived from patients with IPF produced more secreted and cell-associated fibulin-1 than fibroblasts derived from patients without lung disease. This dysregulation was seen at the transcriptional level as fibroblasts from patients with IPF also produced more mRNA under basal conditions than

fibroblasts from subjects without lung disease. The contribution of particular ECM molecules to the development of fibrogenesis has not been extensively investigated in IPF, as research has largely focused on either chemokines or circulating progenitors of myofibroblasts as targets to dampen the "pro-fibrotic environment" that drives fibrosis (Naik et al. 2012). Although it is possible that excessive production of secreted fibulin-1 by the resident fibroblasts of IPF patients contributes to the increased fibulin-1 level found in the blood, a second hypothesis could be that damage to alveolar epithelial cells also causes increased fibulin-1 secretion.

As shown in the ROC curve analysis, serum fibulin-1 has predictive value for acute disease progression in patients with IPF which is an important finding for the future management of IPF. The AUC analysis showed that there was a 71% chance that there was a higher serum fibulin-1 level in a patient with IPF who experienced a progression event within 1 year compared to a patient who remained stable. In addition, patients with a high level of serum fibulin-1 had a higher likelihood of progression and a shorter progression-free survival time compared to their low fibulin-1 counterparts.

A number of serum biomarkers of disease severity and progression in IPF have been identified and reviewed (Richards et al. 2012; Vij and Noth 2012). These include mucin-1 (KL-6), surfactant proteins SP-A and SP-D, matrix metalloproteinases 1 and 7, chemokines CCL18 and CXCL8, calgranulin B (S100A12), intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1) as well as periostin (Naik et al. 2012). Other biomarkers include the gene polymorphism, mucin 5B (MUC5B)(Seibold et al. 2011).

This shows that it is likely that numerous factors contribute to fibrogenesis. The value of our focus on fibulin-1 lies in the fact that we have been able to study it in multiple patient cohorts and multiple forms - soluble and tissue incorporated - and have been able to relate these to physiological measurements of lung function. In addition, while all lung fibroblasts are capable of producing fibulin-1, an increased production of fibulin-1 likely reflects the activated fibrogenic state of fibroblasts derived from patients with IPF.

It is crucial to identify factors which are able to categorise which IPF patients will rapidly decline compared to those patients with a more stable form of the disease. Further follow up studies to confirm the potential of fibulin-1 levels for the stratification of patients with IPF are urgently needed. This information will enable clinicians to optimise early referral to transplantation programs and palliative care. Additionally, fibulin-1 may be a useful tool to stratify patients for inclusion in future therapeutic trials, and to guide future management decisions.

In the future, it would be worthwhile to measure fibulin-1 levels in fibroblasts and tissues isolated from patients with the other ILDs, in particular sarcoidosis, due to its rare fibrotic component. Differences in levels of certain biomarkers between ILDs would help in identify which proteins are surrogate markers of fibrogenesis, not just interstitial lung disease.

# Chapter 3. **Other matrix proteins and fibulin-1**

## 3.1 Introduction

It is the extracellular matrix (ECM) that allows tissue to withstand tensile forces and collagen is the main source of this strength (Canty and Kadler 2005). Fibroblasts are the main source of ECM in the lung (Hutchison et al. 2013) and changes in the ECM can influence the behaviours of the cells that populate it (Klingberg et al. 2013). The role of fibroblasts in pulmonary fibrosis will be investigated in greater detail in Chapter 4. Fibroblasts routinely deposit collagen and other ECM molecules during normal tissue homeostasis but this process becomes uncontrolled during pathological wound healing such as fibrosis (Phan 2002).

The process of collagen and elastin fibril formation from their insoluble components is a complicated process that is outside the scope of this thesis however, the general overview is that the matrix is a collagen scaffold from which a network of interlacing proteins is formed (Bosman and Stamenkovic 2003).

Collagen is the most abundant protein in the ECM, accounting for about 40% of the total protein mass of the ECM, and densely fibrotic tissue display increased type I (Raghow et al. 1985) and type IV collagens (Specks et al. 1995). Collagen has long been the hallmark protein of pulmonary fibrosis (Reiser and Last 1983). Collagen IV was first reported to be increased in the serum of patients with idiopathic pulmonary fibrosis (IPF) in 1996 (Kasuga et al. 1996). It was shown that measurement of serum concentrations would be reflective of fibrotic changes occurring in the lung.

Because the ECM is a complicated and diverse structure, it is likely that there are numerous ECM proteins that are dysregulated in the context of pulmonary fibrosis. In support of this, there are many differences in the composition of decellularized ECM from normal and fibrotic lungs that have recently been described (Booth et al. 2012). The bioactive nature of the ECM, the majority of which is deposited by pathogenic fibroblasts, is increasingly being recognized as a key factor of the fibrogenic process (Klingberg et al. 2013).

Because of this, three other ECM proteins related to fibulin-1 that are also produced by lung fibroblasts were selected for investigation in this study (Figure 3.1). Fibronectin, periostin, and tenascin-C are members of the matricellular class of proteins so named because of their ability to modulate cell-matrix interactions (Frantz et al. 2010). Like other matricellular proteins including fibulin-1, these proteins are typically expressed at high levels during periods of development, but only appear in adult tissue during repair or disease (Strieter 2008; Kaarteenaho et al. 2010; Midwood et al. 2011).

Collagen IV interacts directly with fibulin-1 during development (Kubota et al. 2012) and with plasma fibronectin during infection (Hauk et al. 2008). Collagen I interacts directly with periostin during collagen I fibre formation (Kii et al. 2010), and collagen I increases tenascin C expression in smooth muscle cells (Jones et al. 1999).

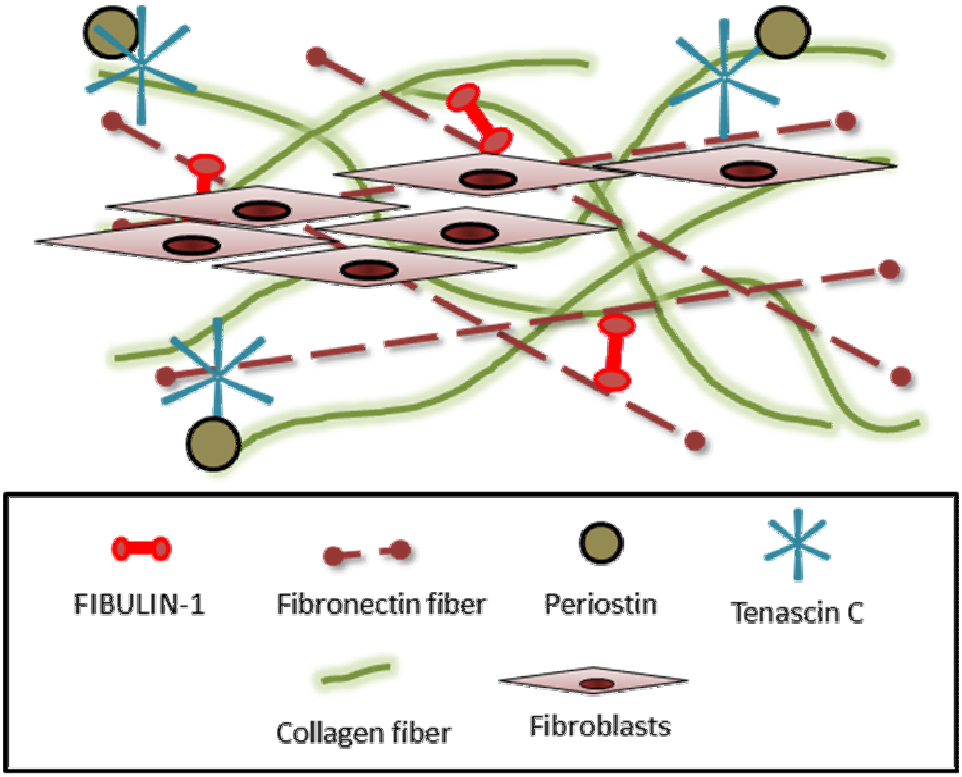



**Figure 3.1 Extracellular matrix molecules produced by lung fibroblasts that were investigated in this thesis**

Fibronectin was the first discovered binding partner of fibulin-1 (Argraves et al. 1989) and is essential in normal tissue maintenance (Tran et al. 1995). It is a large 220-250 kDa molecule that plays a key role in cell migration and adhesion in addition to being the initial scaffold for the ECM. Fibronectin provides the initial "wound plug" during haemostasis, the first stage of wound healing. In association with fibrin, the fibronectin/fibrin clot seals the wound before collagen is deposited by contractile mesenchymal cells (Stenman et al. 1980).

Fibulin-1, which also binds fibrinogen (Tran et al. 1995), is found alongside fibronectin and can inhibit the effect of fibronectin on cell attachment and spreading (Twal et al. 2001). Fibronectin increases cell attachment and spreading through activation of its integrins, which are the main class of matrix receptors that allow cross-talk between cells and the matrix (Bosman and Stamenkovic 2003). Binding sites on fibronectin for other ECM proteins, such as periostin (Kudo 2011) and tenascin-C (Kudo 2011), further demonstrate how complex the relationship between ECM proteins is.

Periostin is a secreted, 90kDa, cell adhesion protein that plays an important role during connective tissue development (Hamilton 2008). Fibroblasts produce significant amounts of periostin (Hamilton 2008), as well as fibulin-1, and immunohistochemical staining shows increased deposition of periostin in fibrotic tissue (Naik et al. 2012). Periostin has been implicated in the sub-epithelial fibrosis of asthma (Takayama et al. 2006) and serum levels of periostin have been shown to predict disease progression in patients with IPF (Naik et al. 2012).

The binding sites for fibronectin, tenascin-C and collagen I are located adjacent to each other on periostin (Norris et al. 2007). This allows periostin to acts as a structural

bridge between the larger fibres and to support the deposition of tenascin-C into the matrix (Kii et al. 2010).

Tenascin-C is member of the tenascin family, which are known to be overexpressed in diseases like asthma and certain cancers (Kaarteenaho-Wiik et al. 2000), although tenascin-C is the only member of the family which forms hexamers. Interestingly, like fibulin-1 and fibronectin before it, tenascin-C is associated with fibrin expression in tissue (Brellier et al. 2011) and it is thought that its hexameric structure allows it to support the larger fibres in the ECM (Kii et al. 2010).

In a role that can be thought of as opposing the actions of fibronectin, tenascin-C is an anti-adhesive molecule (Midwood et al. 2011) which also increases the expression and activity of matrix metalloproteinases (Imanaka-Yoshida 2012). One mechanism driving these processes is shared with fibulin-1. Both tenascin-C and fibulin-1 bind to the HepII domain on fibronectin (Balbona et al. 1992; Williams and Schwarzbauer 2009) and block syndecan-4 (cell surface receptor also implicated in pulmonary fibrosis (Jiang et al. 2010) binding.

Serum tenascin-C (Inoue et al. 2013), serum periostin (Yamaguchi et al. 2013) and serum fibronectin (Lafuma et al. 1987) have been reported to be increased in fibrotic lung conditions, thus supporting the hypothesis that serum ECM molecules can be used as biomarkers of disease progression in pulmonary fibrosis. Although there have been several blood-based biomarkers of disease progression in patients with pulmonary fibrosis reported (Richards et al. 2012) the utility of the aforementioned ECM molecules as biomarkers, as well as their inter-relationships (Table 3.1), has not been fully explored.

**Table 3.1 ECM proteins interact with each other as well as with collagen**

| | Fibronectin | Periostin | Tenascin-C | Collagen |
|---|---|---|---|---|
| Fibulin-1 | Fibulin-1 binds to fibronectin (Argraves et al. 1989) | Fibulin-1 and periostin are both increased in Hodgkin's lymphoma (Kischel et al. 2011) | Fibulin-1 shares adhesion mechanism with tenascin-C (Williams and Schwarzbauer 2009) | Fibulin-1 interacts directly with Col IV (Kobayashi et al. 2007) |
| Fibronectin | | Periostin binds to fibronectin (Takayama et al. 2006) | Tenascin-C binds to fibronectin (Midwood et al. 2011) | Collagen interacts via membrane protein with fibronectin (Stenman et al. 1980) |
| Periostin | | | Tenascin-C structurally supported by periostin (Kii et al. 2010) | Collagen I fibre formation regulated by periostin (Hamilton 2008) |
| Tenascin-C | | | | Collagen I induces tenascin-C (Jones et al. 1999) |

The physical properties of connective tissues are determined by the amount, type and composition of the ECM (Culav et al. 1999). Importantly, it has been shown that the interconnecting ECM proteins such as periostin can act on the biomechanical properties of the tissue they populate (Norris et al. 2007). It is known that the mechanical properties of the lung, similar to any organ, are dependent on the physical formation of the collagen and elastic fibres and that these differences may be due to differences in ECM composition (Antunes et al. 2009).

As shown in Chapter 2, increased levels of fibulin-1 were an accurate indication of disease progression of patients with IPF. It is the aim of this chapter to establish the strength and specificity of that relationship by comparing the performance of fibulin-1 as a biomarker in IPF against the performance of three other ECM proteins known to be implicated in pulmonary fibrogenesis.

This chapter aimed to establish if the relationship between fibulin-1 and pulmonary fibrosis was unique, or if similar pathological associations (such as the ability to predict disease progression in patients with IPF) could be seen in other ECM proteins also thought to be involved in fibrogenesis. In addition, a group of patients with chronic obstructive pulmonary disease (COPD) was included to investigate if ECM dysregulation was a function of any diffuse parenchymal disease.

## 3.2 Methods

### 3.2.1 Patient data

The patients with and without IPF from whom the fibroblasts were derived were previously described in Chapter 2 (Table 2.8). The patients with COPD from whom the fibroblasts were derived are described in Table 3.10.

### 3.2.2 Fibroblast isolation, cell culture and sample collection

Primary parenchymal fibroblasts were cultured and set up for experiment as previously described in Chapter 2 (Methods 2.2.5, 2.2.6, 2.2.7).

### 3.2.3 RNA isolation and QPCR

Primers from Invitrogen were obtained for periostin (Hs01566734_m1), tenascin-C (Hs01115665_m1), and fibronectin (Hs00365052_m1). RNA isolation and QPCR were performed as described in Chapter 2 (Methods 2.2.8).

### 3.2.4 Sandwich ELISA

Cell-secreted periostin, tenascin-C and fibronectin were measured by enzyme-linked immunosorbent assay (ELISA) antibody sets (for periostin and tenascin-C) or complete kits (for fibronectin). The specifics of each assay are summarised in Table 3.2. For fibronectin, all reagents were provided as part of the kit (Life Science cat# L00170025).

The ELISA assay was similar for each of the ECM proteins. For periostin and tenascin-C, polystyrene 96-well Corning COSTAR flat-bottom plates (Fisher Scientific, Sydney) were coated with 100µL/well of the primary antibody of interest

diluted in phosphate buffered saline (PBS pH7.2, 137mM NaCl, 2.7mM KCl, 100mM $Na_2HPO_4$, 2mM $KH_2PO_4$) as summarised in Table 3.2. The plates were sealed with Parrafilm (Holgate Scientific, NSW) and incubated overnight at 4°C. The primary antibody was then decanted on clean paper towels and each well was washed three times with wash buffer (0.05%v/v Tween-20 in PBS). After washing, 300µL of 1%BSA/PBS (blocking solution) was added to each well and the plates were sealed and incubated for one hour at room temperature on a shaker. For fibronectin, the plates provided as part of the kit were pre-coated with the primary antibody and samples were assayed immediately.

Plates coated with anti-periostin and anti-tenascin-C antibodies were decanted and washed three times as before and 100µL of cell-free supernatant (containing only secreted extracellular proteins) was added to each well in duplicate. Standards for all three ECM proteins were diluted in a serial two-fold manner with the top standard concentrations for each assay listed in Table 3.2. Wells with standards also contained 100µL. The final standard concentration was the respective antibody diluents without any standard protein present. In all plates, a sample of cell-free culture media was also added as a baseline control for the endogenous level of ECM protein.

After incubation for two hours at room temperature on a shaker (periostin, tenascin-C) or 90 minutes at 37°C (fibronectin), the plates were again decanted and washed as before and 100µL/well of the secondary antibody was added and plates were incubated at room temperature as described in Table 3.2. Plates were again decanted and washed as before and incubated with 100µL/well streptavidin-horseradish peroxidase (HRP) diluted in 1% BSA/PBS and incubated as described in Table 3.2.

Lastly, plates were decanted and washed and 50µL of the substrate solution 3,3',5,5'-Tetramethylbenzidine (TMB) (Zymed, CA, USA) was added to each well. Plates were incubated in the dark for 5-15 minutes (dependent on colour development) and the reaction was stopped with the addition of 50µL/well of stop solution (0.1M $H_3PO_4$) per well. Plates assayed for fibronectin used TMB and stop solution provided as part of the kit. The optical density (OD) was read at 450nm using a microplate reader and the amount of each ECM protein was determined from the OD of the corresponding standard curves.

**Table 3.2 Concentrations of the materials used in secreted ECM measurements**

| | **Periostin kit** (R&D cat#DY2955) | **Tenascin-C** (R&D cat# MAB3358, BAF3358) | **Fibronectin kit** (Life Science cat# L00170025) |
|---|---|---|---|
| Primary antibody | 4µg/mL in PBS | 1µg/mL in PBS | Pre-coated plates |
| Blocking media | 1%BSA/PBS | 1%BSA/PBS | Not applicable |
| Standard | 6000pg/mL → 0pg/mL | 30ng/mL → 0ng/mL (R&D cat#3358-TC) | 100ng/mL → 0ng/mL |
| Samples | Diluted as necessary in PBS | Diluted as necessary in PBS | Diluted as necessary in PBS |
| Secondary antibody | 0.4µg/mL in PBS | 0.5µg/mL in PBS | Kit reagents |
| Detection antibody | 1:200 (R&D cat#DY998) | 1:200 (R&D cat#DY998) | Kit reagents |
| Substrate | TMB (Zymed cat#00-2023) | TMB (Zymed cat#00-2023) | Kit reagents |
| Stop solution | 1M $H_3PO_4$ | 1M $H_3PO_4$ | Kit reagents |
| Lower limit of detection | 93.8pg/mL | 0.47ng/mL | 0.72ng/mL |

### 3.2.5 Immunohistochemistry, image capture and analysis

Immunohistochemistry was performed in the same fashion as for fibulin-1 and described in Chapter 2 (Methods 2.2.12). Like fibulin-1, sections that were investigated for the levels of periostin and fibronectin did not require antigen retrieval. Antibodies directed against both periostin and fibronectin were produced from rabbit and therefore used the same isotype control (Table 3.3).

In addition, because the same DAB system was used for visualisation (2.2.12) the same image capture and deconvolution techniques were employed for both periostin and fibronectin quantification (2.2.13, 2.2.14).

**Table 3.3. Concentrations of the primary antibodies used for immunohistochemistry**

| | Source | Final concentration |
|---|---|---|
| Periostin antibody | ABCAM (cat# ab14041) | 0.5µg/mL |
| Fibronectin antibody | Sigma (cat# F3648) | 0.5µg/mL |
| Rabbit IgG isotype control | Dako | 0.5µg/mL |

### 3.2.6 Statistical Analysis

Graphs were made using GraphPad Prism 6 Software for Windows (Version 6 GraphPad Software Inc. 1992-2007). Statistical analysis was done using SPSS (Version 21 IBM Corporation 1989-2012).

Distributions of serum, parenchyma and fibroblast levels of periostin, tenascin-C and fibronectin, and lung function parameters were carried out in the same fashion as described in Chapter 2 (Methods 2.2.15).

It was not necessary to determine the repeatability and reliability of the periostin, tenascin-C or fibronectin measurements as these investigations were carried out by sandwich ELISA and a standard curve was used in every experiment for quantification.

Between-group differences, receiver-operating characteristic (ROC) curves, and correlations between serum and tissue ECM proteins and lung function parameters were performed as described in Chapter 2 (Methods 2.2.15).

Because the same patients were investigated for the levels of fibulin-1 and collagen (Chapter 2), as well as for periostin, tenascin-C and fibronectin (Chapter 3), correlation analysis investigating the relationship between ECM proteins was possible. Correlations between fibulin-1, periostin, tenascin-C and fibronectin in the serum of patients with interstitial lung disease (ILD) were performed with Spearman's rank correlation co-efficients.

Serial sections were used to investigate the levels of fibulin-1, collagen, periostin and fibronectin in patients with IPF. This allowed for additional correlation analysis between the ECM proteins in parenchyma tissue to be performed.

Statistical analysis was performed as described in Chapter 2 (Methods 2.2.15).

## 3.3 Results

### 3.3.1 Serum periostin is increased in patients with IPF

Serum periostin was increased in patients with IPF compared to subjects without lung disease and patients with other ILDs after adjustments for potential confounding variables – age, gender and smoking history. Unlike serum fibulin-1, serum periostin was not increased in patients with IPF compared to patients with sarcoidosis (Figure 3.2).

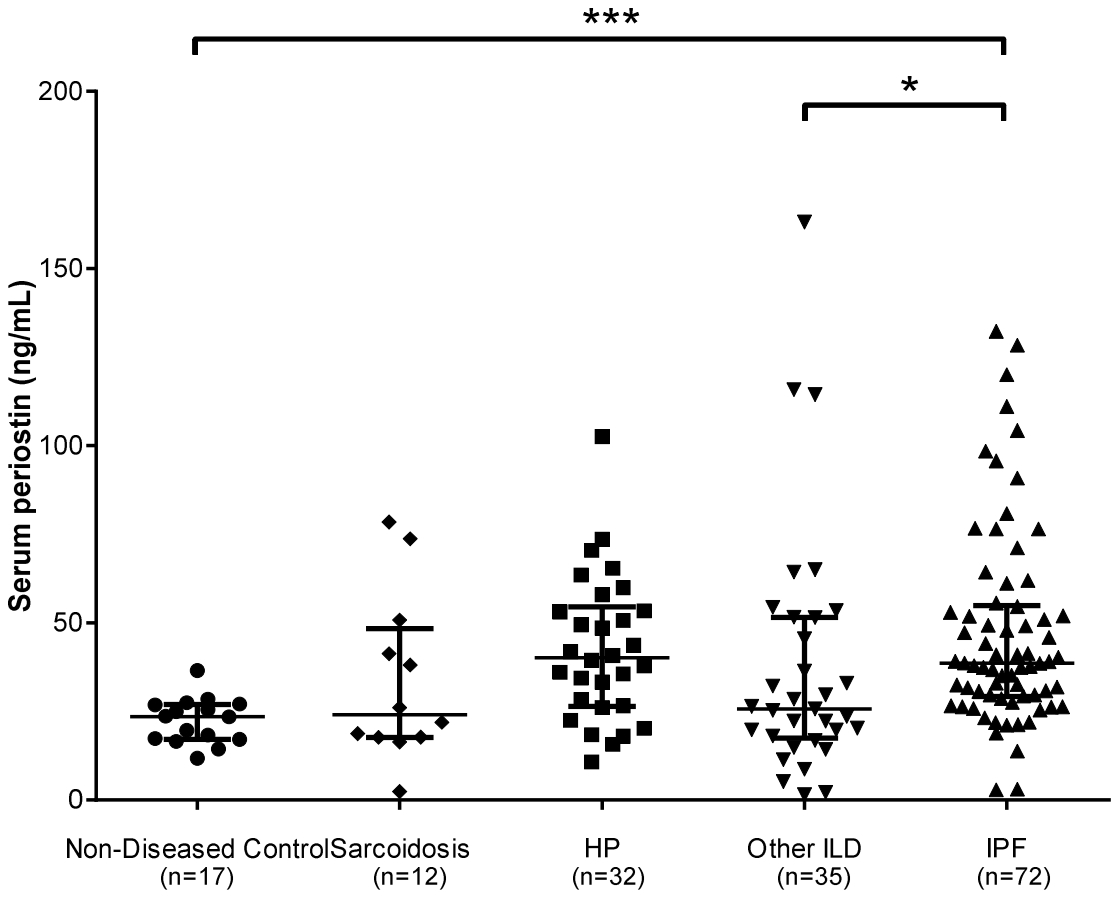



**Figure 3.2 Serum periostin is increased in patients with IPF compared to subjects without lung disease.**
Serum periostin levels were measured by ELISA. Values were adjusted for age, gender and smoking history. (ANCOVA, n=168, post-test Tukey's *p<0.05. ***p=0.006, median ± 25th & 75th percentiles).
"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).
HP hypersensitivity pneumonitis, ILD interstitial lung disease, IPF idiopathic pulmonary fibrosis, ANCOVA analysis of covariance

### 3.3.2 Serum tenascin-C is decreased in patients with IPF compared to patients with HP

Interestingly, the level of tenascin-C was decreased in the patients with IPF compared to patients with HP (Figure 3.3).

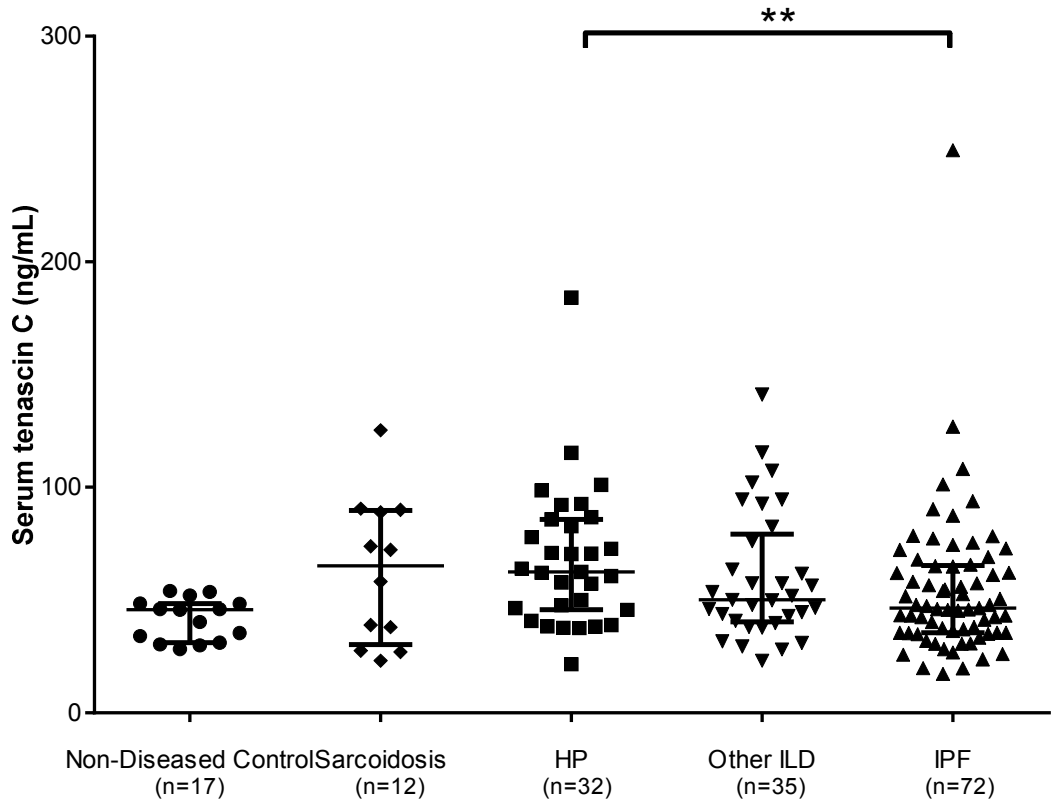



**Figure 3.3 Serum tenascin-C is decreased in patients with IPF compared to patients with HP**
Serum tenascin-C levels were measured by ELISA. Values were adjusted for age, gender and smoking history. (ANCOVA, n=168, post-test Tukey's **p<0.01, median ± 25th & 75th percentiles).
"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).
HP hypersensitivity pneumonitis, ILD interstitial lung disease, IPF idiopathic pulmonary fibrosis, ANCOVA analysis of covariance

### 3.3.3 Serum fibronectin is not increased in patients with ILD

There was no difference in serum fibronectin level between the 5 groups (Figure 3.4).

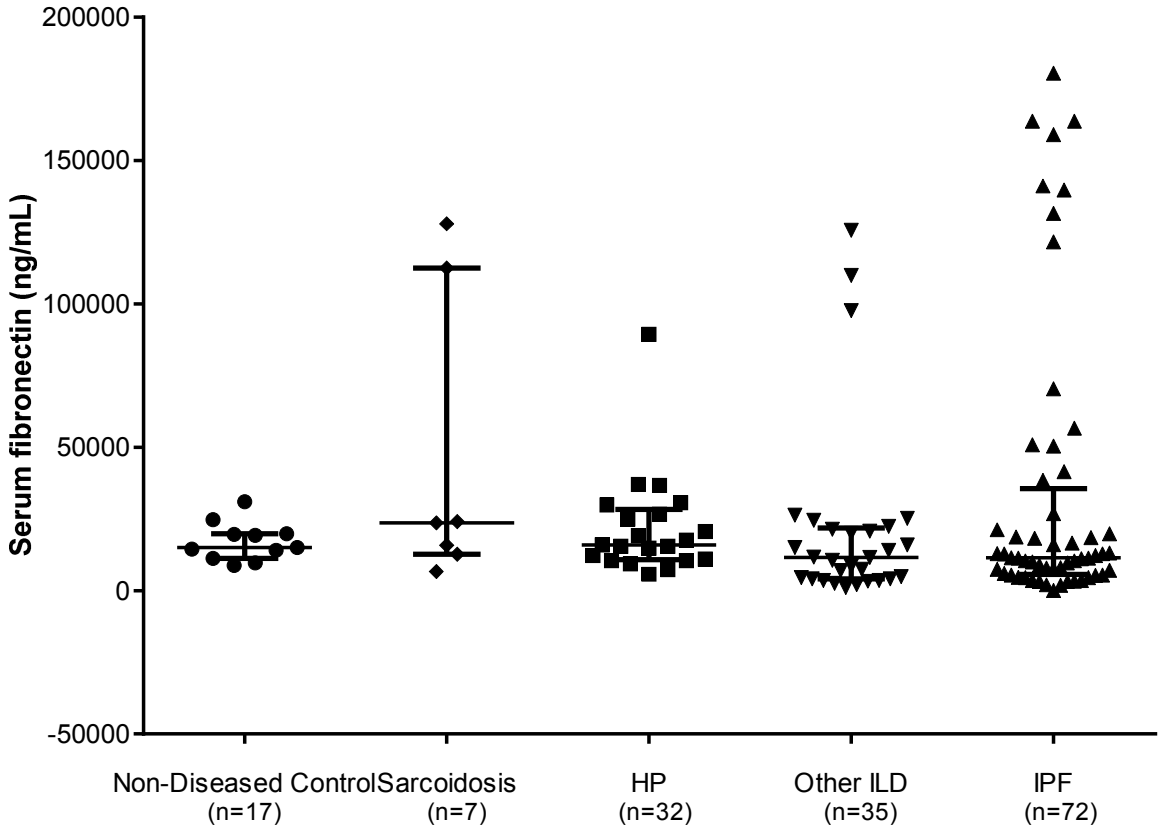



**Figure 3.4 Serum fibronectin is not increased in patients with ILD**
Serum fibronectin levels (ELISA) were adjusted for age, gender and smoking history. (ANCOVA, n=168, median ± 25th & 75th percentiles).
"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).
HP hypersensitivity pneumonitis, ILD interstitial lung disease, IPF idiopathic pulmonary fibrosis, ANCOVA analysis of covariance

### 3.3.4 Serum periostin and tenascin-C correlate with lung function in patients with ILD

Next the relationship between the serum level of the three ECM proteins and lung function in patients with ILD was examined. Serum draw and lung function measurements were taken no more than 15 (±5) days apart.

Both fibulin-1 (Table 2.5) and periostin correlated significantly with poorer percent predicted FVC (FVC%), percent predicted $DL_{CO}$ ($DL_{CO}$%), composite physiologic index (CPI) and percent predicted TLC (TLC%) measurements(Table 3.4) in patients with ILD. Tenascin-C only correlated with FVC% while fibronectin did not correlate with any measurement (Table 3.4).

None of the four ECM proteins examined correlated with age, gender or smoking history.

**Table 3.4 Serum ECM proteins correlate with lung function in patients with ILD**

| | Periostin | Tenascin-C | Fibronectin |
|---|---|---|---|
| $FEV_1$, % predicted | -0.091 | -0.236 | 0.039 |
| FVC, % predicted | -0.265* | -0.287* | -0.006 |
| $DL_{co}$, % predicted | -0.308* | -0.178 | 0.189 |
| CPI, units | 0.377** | 0.213 | -0.091 |
| TLC, % predicted | -0.312* | -0.225 | -0.083 |
| Age, yr | 0.131 | 0.172 | 0.132 |
| Gender | 0.031 | 0.043 | 0.103 |
| Smoking History | 0.124 | -0.104 | 0.140 |

Lung function parameters and demographic information was obtained for 73 patients with interstitial lung disease (ILD). The time between serum collection and lung function measurements was no more than 15 days (±5). Serum periostin, tenascin-C and fibronectin levels were measured by ELISA. Continuous variables were analysed with spearman's rank correlation analysis and categorical variables were analysed by Pearson chi-square test.
*p<0.05 ,**p<0.01

### 3.3.5 Serum ECM proteins correlate with each other in patients with IPF

Serum fibulin-1 and periostin both correlated strongly with tenascin-C in patients with IPF (Table 3.5). In correlation analysis, a coefficient of more than 0.3 is considered a "large" effect and is therefore more likely to be biologically meaningful (Hayek and Heyer 2005).

**Table 3.5 The correlation between the four serum ECM proteins is disease group specific**

| **Sarcoidosis** (n=12) | **Fibulin-1** | **Periostin** | **Tenascin-C** | **Fibronectin** |
|---|---|---|---|---|
| Fibulin-1 | | .126 | -.119 | .000 |
| Periostin | .126 | | .329 | -.679 |
| Tenascin-C | -.119 | .329 | | -.536 |
| Fibronectin | 0.000 | -.679 | -.536 | |

| **HP** (n=32) | **Fibulin-1** | **Periostin** | **Tenascin-C** | **Fibronectin** |
|---|---|---|---|---|
| Fibulin-1 | | .188 | .285 | .020 |
| Periostin | .188 | | .017 | -.468* |
| Tenascin-C | .285 | .017 | | .355 |
| Fibronectin | .020 | -.468* | .355 | |

| **"Other " ILD** (n=35) | **Fibulin-1** | **Periostin** | **Tenascin-C** | **Fibronectin** |
|---|---|---|---|---|
| Fibulin-1 | | .078 | .163 | -.001 |
| Periostin | .078 | | .341 | .312 |
| Tenascin-C | .163 | .341 | | .019 |
| Fibronectin | -.001 | .312 | .019 | |

| **IPF** (n=72) | Fibulin-1 | Periostin | Tenascin-C | Fibronectin |
|---|---|---|---|---|
| Fibulin-1 | | .212 | .416** | .126 |
| Periostin | .212 | | .406** | -.189 |
| Tenascin-C | .416** | .406** | | -.232 |
| Fibronectin | .126 | -.189 | -.232 | |

Serum fibulin-1 was measured by western blot and quantified using densitometric analysis. Serum periostin, tenascin-C and fibronectin levels were measured by ELISA.
Spearman's rank correlation analysis (*p<0.05, **p<0.01)
"Other" refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).
ECM extracellular matrix, HP hypersensitivity pneumonitis, ILD interstitial lung disease, IPF idiopathic pulmonary fibrosis

### 3.3.6 No difference in serum periostin, tenascin-C or fibronectin between patients with IPF who progressed and those patients who remained stable

There was no difference in serum ECM level between those patients with IPF who experienced significant lung function decline (≥ 10% fall in FVC%, ≥ 15% fall in $DL_{CO}$%, or death) during the year following blood draw and those patients who remained stable in the same time period (Table 3.6).

**Table 3.6 Serum periostin, tenascin-C or fibronectin levels in patients with IPF who progressed or who remain stable**

| | Stable (n=21) | | Progressed (n=27) | | |
|---|---|---|---|---|---|
| (ng/mL) | Mean | SD | Mean | SD | P Value |
| Periostin | 54.26 | 39.03 | 48.46 | 27.39 | .549 |
| Tenascin-C | 46.20 | 18.05 | 60.66 | 43.95 | .173 |
| Fibronectin | 14916.43 | 18669.12 | 23920.83 | 46436.72 | .481 |

Patients with IPF were followed up for at least one year (365 ± 1 day) following blood draw.
Serum periostin, tenascin-C and fibronectin levels were measured by ELISA.
Between group differences were assessed by unpaired t-tests. All p values were > 0.05 and therefore not considered significant.
IPF idiopathic pulmonary fibrosis, SD standard deviation

### 3.3.7 Periostin, tenascin-C, fibronectin do not predict disease progression in patients with IPF

In order to investigate the utility of each ECM protein as a biomarker of disease progression, ROC curves were constructed (Figure 3.5).

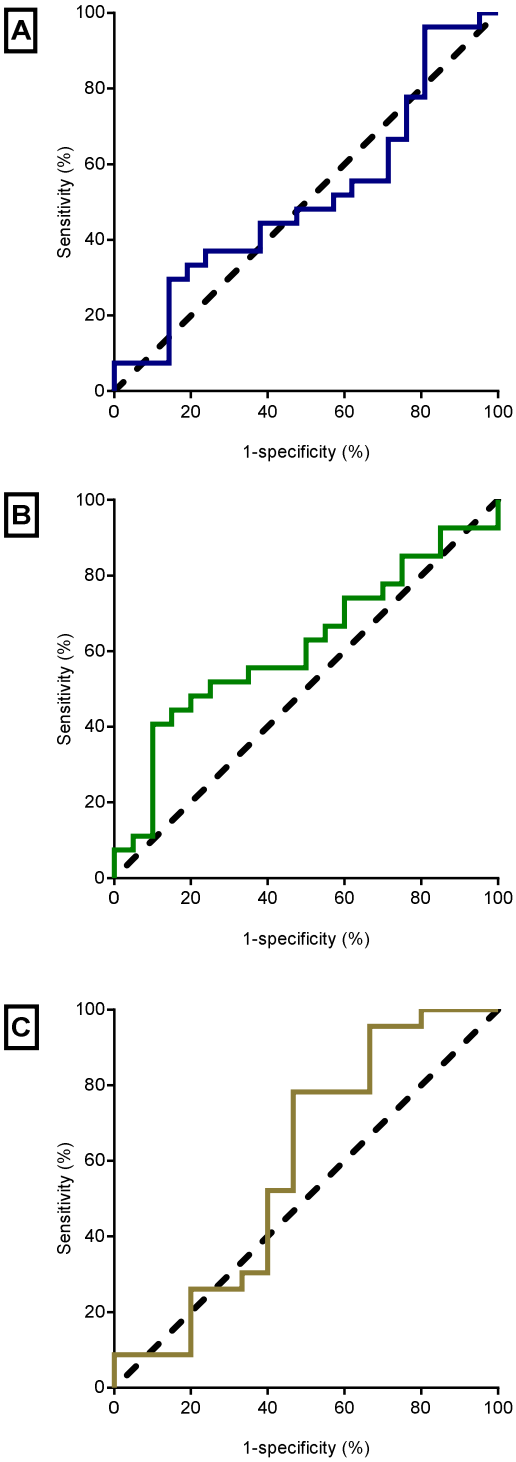



**Figure 3.5 ROC analysis shows that neither periostin, tenascin-C or fibronectin are able to discriminate disease progression in patients with IPF.**
The utility of periostin (A), tenascin-C (B) and fibronectin (C) as biomarkers of disease progression were modelled using receiver-operating characteristic curve analysis in patients with idiopathic pulmonary fibrosis (IPF) (n=48).

The area under the curve for each of the three potential biomarkers is shown in Table 3.7. Serum periostin, tenascin-C and fibronectin levels were unable to accurately identify patients with IPF who progressed.

**Table 3.7 ROC curve analysis of periostin, tenascin-C and fibronectin**

| ROC Analysis | Area under the curve | 95% Confidence Interval | P value |
|---|---|---|---|
| Periostin | .487 | .319 to .655 | .876 |
| Tenascin-C | .613 | .450 to .776 | .189 |
| Fibronectin | .591 | .389 to .794 | .347 |

Patients with IPF were followed up for at least one year (365 ± 1 day) following blood draw. Serum periostin, tenascin-C and fibronectin levels were measured by ELISA. Serum levels in patients who progressed (n=27) were compared to the levels in patients who did not progress (n=21). A receiver operating characteristic (ROC) curve was generated and the area under the curve was calculated.

### 3.3.8 Tissue periostin and fibronectin levels in patients with IPF

Periostin and fibronectin have been reported to be increased in lung tissue of patients with IPF. As shown in Chapter 2, tissues from patients with IPF showed increased levels of fibrosis as measured by total collagen deposition (Figure 2.9). In addition to having higher levels of fibulin-1 (Figure 2.10), tissue from patients with IPF also had increased levels of periostin compared to tissue from subjects without lung disease (Figure 3.6A). Levels of fibronectin were not different between tissue from patients with IPF and subjects without lung disease (Figure 3.6B).

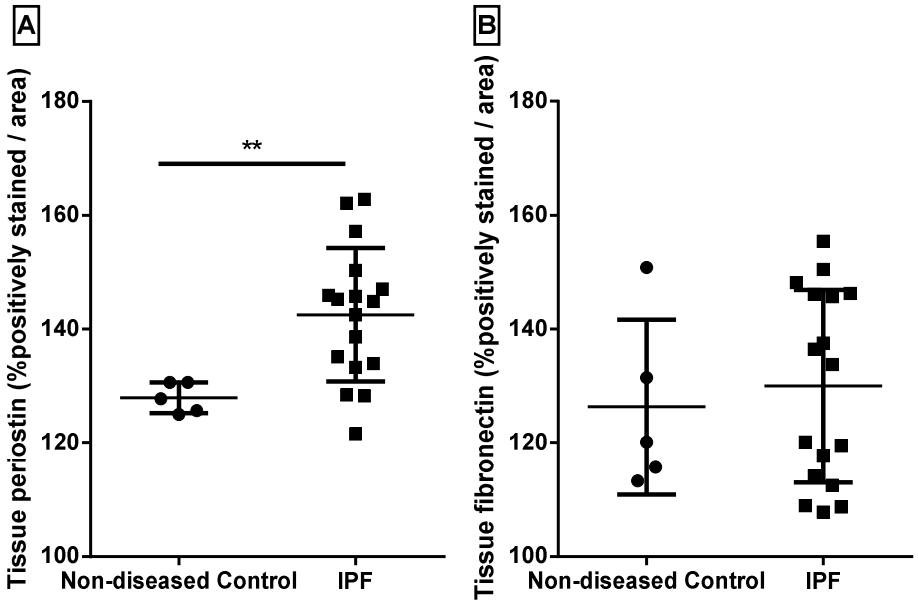



**Figure 3.6 Levels of tissue periostin and fibronectin in patients with IPF and subjects without lung disease**

Levels of (A) tissue periostin and (B) tissue fibronectin were measured by immunohistochemistry. Paraffin-embedded formalin fixed tissue sections from 5 subjects without lung disease and 17 patients with IPF were stained for the proteins individually and each was counterstained for all other tissue components.

Levels of each protein were quantified separately by computer aided image analysis using ImageJ and reported as the area positively stained for each protein as a percentage of the total area stained. Twenty images of each tissue section were analysed, 2-6 tissue sections were measured for each subject. Subsequently, measurements of each image were averaged together to obtain a single value per patient.

Between group differences were compared using the unpaired t-test (****$p<0.0001$, **$p<0.01$).

IPF = idiopathic pulmonary fibrosis

Representative images of tissue from a subject without lung disease (A-D) and from a patient with IPF (E-H) are found in Figure 3.7.

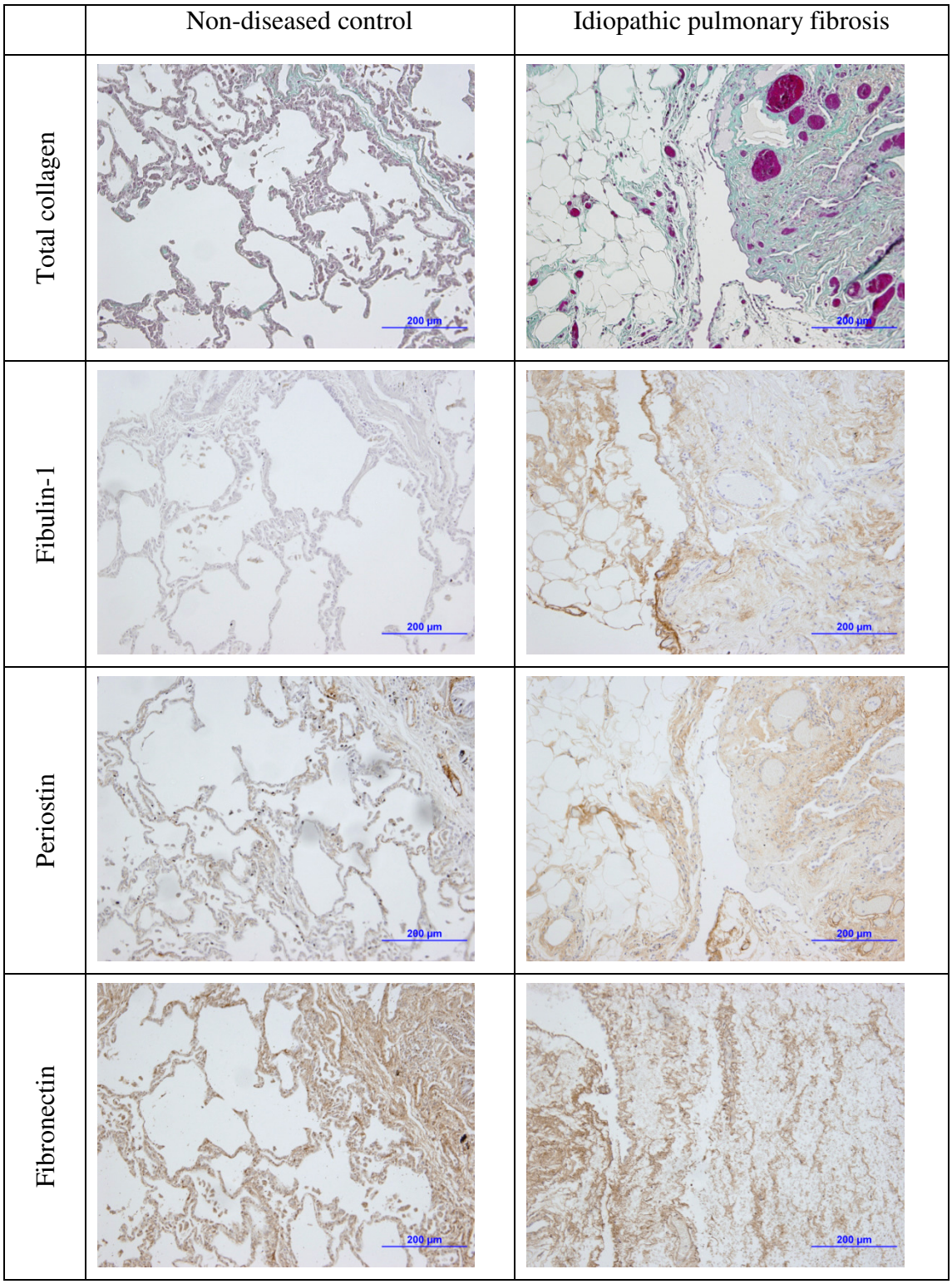



**Figure 3.7 Immunohistochemical stains of total collagen, fibulin-1, periostin, and fibronectin.**
Representative paraffin-embedded formalin fixed parenchymal tissue sections from a subject without lung disease or a patient with IPF were stained for total collagen (green, with cell cytoplasm in red), fibulin-1 periostin or fibronectin (brown). Cell nuclei are blue in fibulin-1, periostin and fibronectin stains.

### 3.3.9 Tissue levels of total collagen, periostin, or fibronectin do not correlate with lung function in patients with IPF

The relationship between the level of tissue ECM proteins and lung function in patients with IPF who had undergone a diagnostic biopsy within 30 (±5) days of a lung function test was then investigated. Unlike tissue fibulin-1, tissue periostin did not correlate with either FEV%1 or FVC% (Figure 3.8). In addition, the relationship between the ECM proteins and other lung function variables was also examined in Table 3.8. Age did not correlate with any ECM protein level.

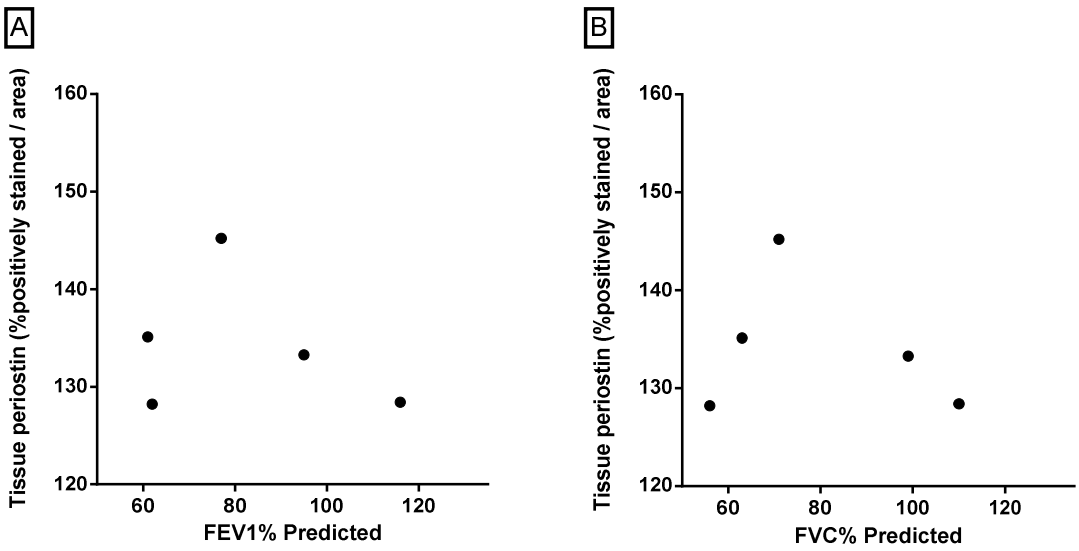



**Figure 3.8 Tissue periostin does not correlate with percentage predicted $FEV_1$ or FVC in patients with IPF**

Paraffin-embedded formalin fixed tissue sections were stained for periostin and counterstained for all other tissue components. Levels of periostin were quantified by computer aided image analysis using ImageJ and reported as the area positively stained for periostin as a percentage of the total area stained. Twenty images from each tissue section were analysed and 2-6 tissue sections were measured for each subject. Averaged tissue level of periostin for each patient (n=5) was compared to their (A) $FEV_1$ and (B) FVC percentage predicted measurements using Pearson product-moment coefficients.
Time between biopsy and lung function measurements were within 30 days (±5).

$FEV_1$ forced expiratory volume in 1 second, FVC forced vital capacity, IPF idiopathic pulmonary fibrosis

**Table 3.8 Tissue ECM protein levels do not correlate with lung function measurements or age in patients with IPF**

| | $FEV_1$% | FVC% | $DL_{co}$% | CPI | TLC% | Age, Yrs |
|---|---|---|---|---|---|---|
| Total Collagen | -.169 | -.178 | -.215 | .226 | .208 | .003 |
| Periostin | -.253 | -.249 | -.225 | .245 | -.034 | .045 |
| Fibronectin | -.564 | -.491 | -.518 | .522 | -.447 | -.067 |

Two to six surgical lung biopsies and lung function parameters were obtained from each patient with idiopathic pulmonary fibrosis (n=5-7). Tissue ECM protein level was measured by immunohistochemistry. Twenty images of each tissue section were analysed and 2-6 tissue sections were measured for each subject. The relationship between tissue ECM level and lung function variables were examined by Pearson correlation analysis and P>0.05 all cases. The time between biopsy and lung function measurements were within 30 days (±5).
$FEV_1$% percentage predicted forced expiratory volume in 1 second, FVC% percentage predicted forced vital capacity, $DL_{CO}$% percentage predicted diffusing capacity of carbon monoxide, TLC% percentage predicted total lung capacity, CPI composite physiologic index, IPF idiopathic pulmonary fibrosis, Yrs years, ECM extracellular matrix

Finally, the relationships between ECM proteins continued into the tissue. Fibulin-1 correlated strongly with periostin with 26.4% (Pearson $r^2 = 0.264$, $p<0.05$) with one protein (eg. Fibulin-1) able to predict the other (eg. Periostin). Tissue periostin correlated even more strongly with fibronectin and collagen as it was able to predict 52.9% of the level of tissue fibronectin (Pearson $r^2 = 0.529$, $p<0.01$) and 66.6% of total collagen (Pearson $r^2 = 0.666$, $p<0.01$) (Figure 3.9, Table 3.9).

**Table 3.9 Tissue ECM proteins correlate with each other in patients with IPF**

| | Fibulin-1 | Periostin | Fibronectin | Total Collagen |
|---|---|---|---|---|
| Fibulin-1 | | 0.514* | 0.479 | 0.286 |
| Periostin | | | 0.727*** | 0.816*** |
| Fibronectin | | | | 0.251 |
| Total collagen | | | | |

Two to six surgical lung biopsies and lung function parameters were obtained from each patient with idiopathic pulmonary fibrosis (IPF) (n=17). Tissue ECM protein levels was measured by immunohistochemistry. Twenty images of each tissue section were analysed and 2-6 tissue sections were measured for each subject. The relationships between tissue ECM levels were examined by Pearson correlation analysis. (*p<0.05, ***p<0.001)
ECM extracellular matrix

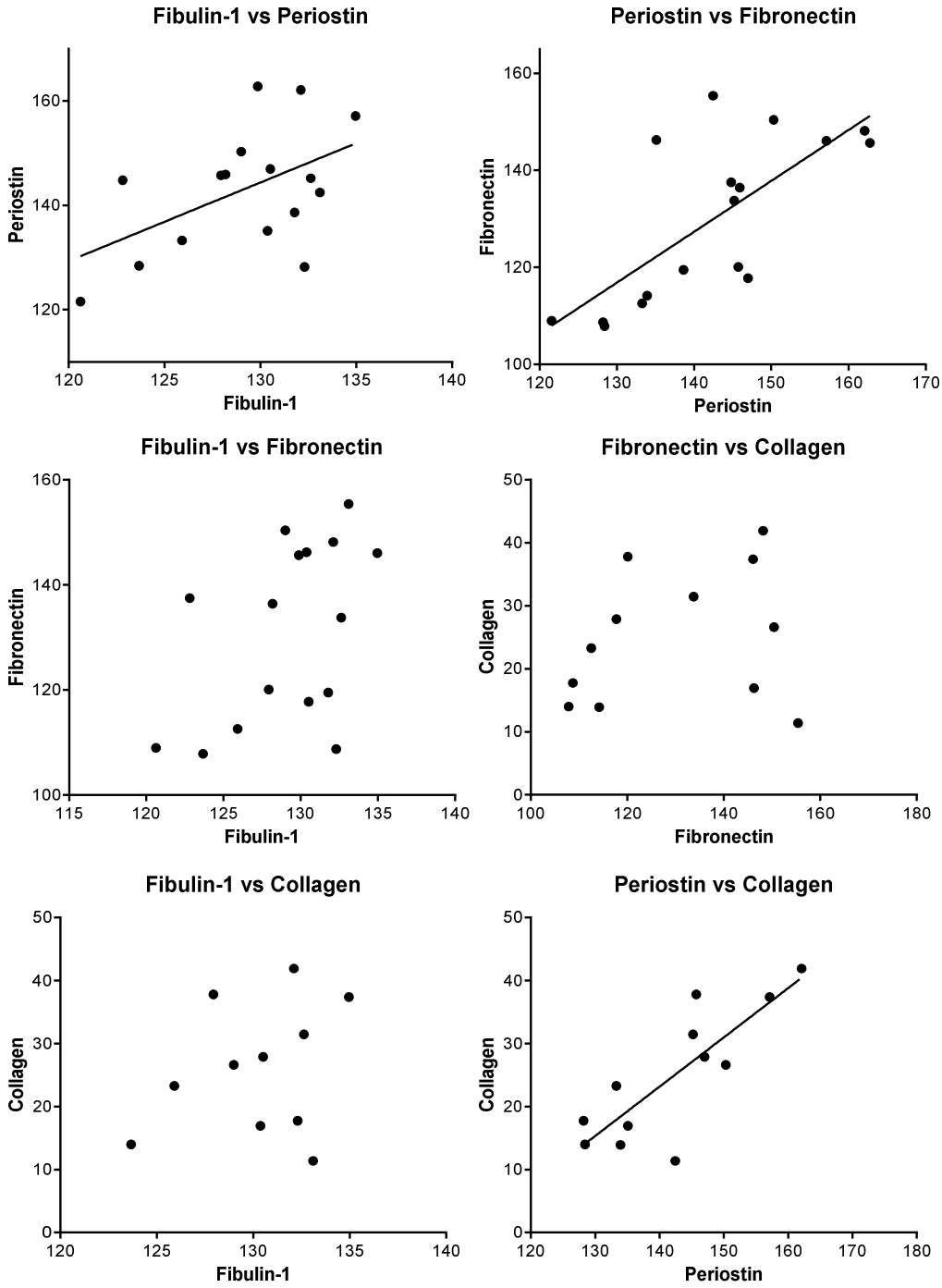



**Figure 3.9 Tissue ECM proteins correlate with each other in patients with IPF**
Paraffin-embedded formalin fixed tissue sections from 17 patients with IPF were stained for each ECM protein and counterstained for all other tissue components. Levels of ECM proteins were quantified by computer aided image analysis using ImageJ and reported as the area positively stained for ECM protein as a percentage of the total area stained. Twenty images of each tissue section were analysed, 2-6 tissue sections were measured for each subject. Subsequently, measurements of each image were averaged together to obtain a single value per patient and shown by a single solid dot. Significant ($p<0.05$) Pearson's product moment correlations are shown with the linear regression line.

For ease of comparison, the fibulin-1 levels in fibroblasts derived from patients with and without IPF have been repeated from Chapter 2 in the following sections.

### 3.3.10 Fibroblasts from patients with IPF do not produce more periostin or fibronectin mRNA

Information of the patients from whom the non-IPF and IPF fibroblasts were derived are found in Table 2.8. Information of the patients from whom the COPD fibroblasts were derived are found in Table 3.10.

**Table 3.10 Patient information from whom COPD fibroblasts were derived**

| Donor # | Gender | Age | Diagnosis | Reason for Surgery |
|---|---|---|---|---|
| COPD 1 | Male | 62 | COPD | Transplant |
| COPD 2 | Male | 54 | COPD | Transplant |
| COPD 3 | Male | 63 | COPD | Transplant |
| COPD 4 | Male | 58 | COPD | Transplant |

Parenchymal fibroblasts were isolated from lung tissue obtained from donors undergoing transplantation. Pulmonary function and smoking data were not available. COPD chronic obstructive pulmonary disease

Figure 3.10 shows the mRNA levels of the four ECM proteins of interest in this thesis. For ease of reference, results of fibulin-1 mRNA in non-IPF and IPF fibroblasts are repeated from Figure 2.12.

Tenascin-C mRNA production was significantly lower in fibroblasts from IPF patients compared to those from patients with COPD and subjects without lung disease. Fibulin-1 mRNA was the only one of the ECM proteins to be increased in fibroblasts from patients with IPF compared to patients without IPF

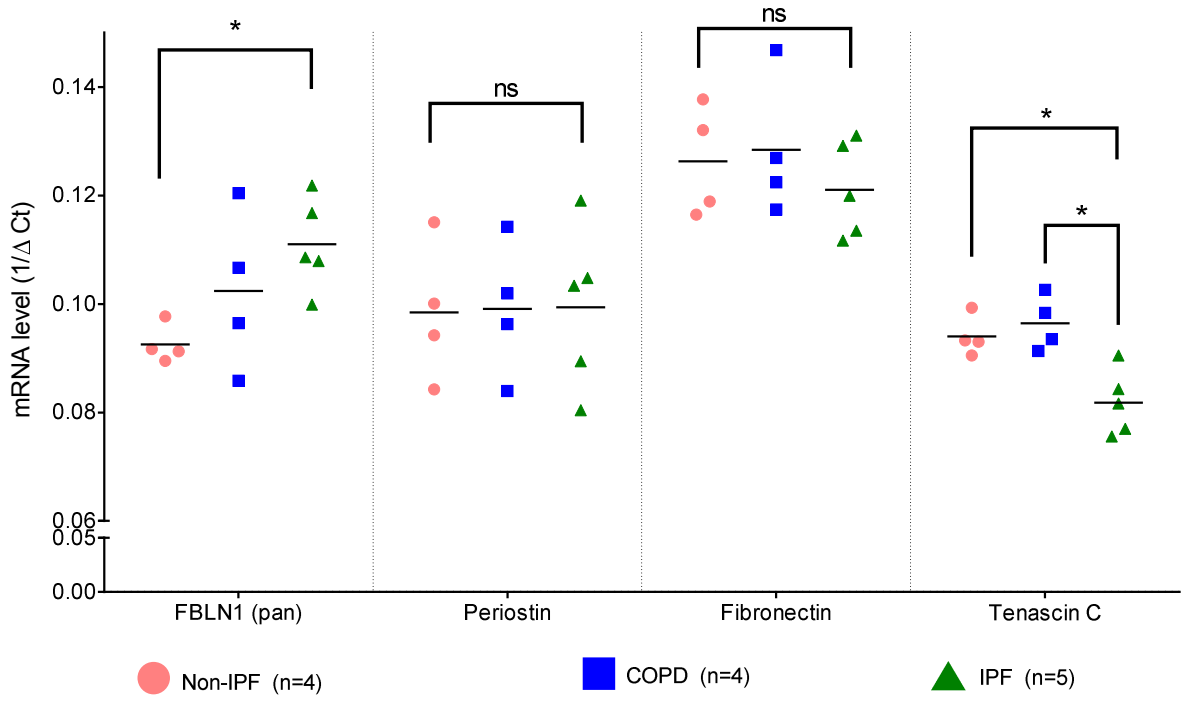



**Figure 3.10 Basal mRNA levels of ECM proteins in fibroblasts.**

Primary parenchymal fibroblasts from patients with IPF (n=5) and age and gender matched patients without IPF (n=4) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep, for 24 hours and maintained in fresh 0.1% FBS/DMEM/1% pen-strep for a further 72 hours. Non-IPF fibroblasts are derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. 500 ng of mRNA was converted to cDNA for this comparison. *p<0.05 one way ANOVA with Tukey's post-test for between group comparisons.
Data are expressed as 1/delta cycle threshold to 18S (ΔCt) to enable a greater number to reflect more mRNA.

FBLN1 fibulin-1, COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

### 3.3.11 Basal production of fibulin-1, periostin and tenascin-C by fibroblasts

To investigate a possible source of serum periostin and tenascin-C the basal levels of the two additional proteins secreted by the same fibroblasts used in Chapter 2 (Figure 2.13) were measured. In addition, the levels of cell-secreted fibulin-1, periostin, and tenascin-C from fibroblasts derived from patients with COPD was also measured. Only fibulin-1 was increased in fibroblasts from patients with IPF compared to subjects without lung disease and patients with COPD (Figure 3.11).

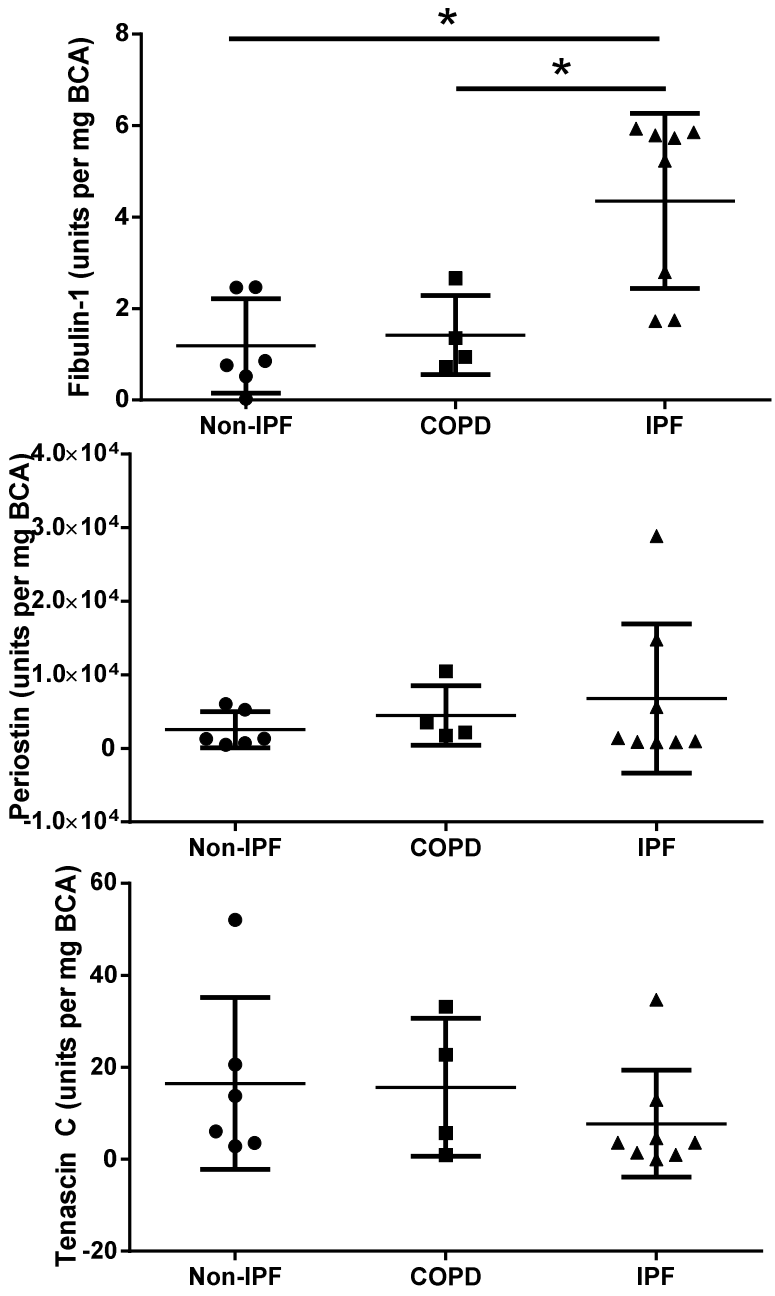



**Figure 3.11 Basal production of secreted ECM proteins by primary fibroblasts**

Primary parenchymal fibroblasts from patients with IPF (n=8) and age and gender matched patients without IPF (n=7) and patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and maintained in fresh 0.1% FBS/DMEM/1% pen-strep for a further 72 hours. Supernatants were collected and analysed by western blot (fibulin-1) and ELISA (periostin, tenascin-C). All values were normalised to total protein of the cell monolayer as measured by BCA assay. Between group differences were assessed by ANOVA with Tukey's post-test analysis *$p<0.05$. Values are expressed as median and interquartile range.
Non-IPF fibroblasts are derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma.
COPD chronic obstructive pulmonary disease, ECM extracellular matrix, BCA Bicinchroninic Acid, IPF idiopathic pulmonary fibrosis, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

### 3.3.12 Summary of results from biomarker investigation

The aim of this chapter was to compare the utility of three other ECM proteins that have been implicated in disease progression of patients with pulmonary fibrosis and are also ECM proteins that relate (eg. bind same proteins) to fibulin-1. Fibulin-1 stood out as a better biomarker of disease progression in patients with IPF compared to periostin, tenascin-C and fibronectin. Fibulin-1 was increased in all the patient materials that were examined in this study and successfully predicted disease progression in patients with IPF, independent of baseline lung function measurements. A summary of the findings covered so far in this thesis is found in Table 3.11.

**Table 3.11 Summary of the biomarker investigation**

| Research Question | | Fibulin-1 | Periostin | Fibronectin | Tenascin-C |
|---|---|---|---|---|---|
| 1 | Is the biomarker increased in the serum of patients with IPF compared to controls? | Yes | Yes | No | No |
| 2 | Does the biomarker predict progression in patients with IPF? | Yes | No | No | No |
| 3 | Is the biomarker increased in the tissue of patients with IPF compared to controls? | Yes | Yes | No | |
| 4 | Do fibroblasts from patients with IPF produce increased mRNA of the biomarker? | Yes | No | No | No |
| 5 | In patients with IPF, do fibroblasts produce increased levels of the soluble biomarker protein? | Yes | No | | No |
| 6 | In patients with IPF, do fibroblasts produce increased levels of the cellular biomarker protein? | Yes | | | |

Grey boxes indicate research questions outside the scope of this thesis.
mRNA messenger ribonucleic acid, IPF idiopathic pulmonary fibrosis

## 3.4 Discussion

Because there are likely to be many ECM proteins that are dysregulated during pathogenic fibrogenesis, three additional ECM proteins were investigated in this Chapter to elucidate if they also were biomarkers of disease progression in patients with IPF. This chapter demonstrates that the ECM proteins, periostin, tenascin-C and fibronectin were not biomarkers of disease progression in patients with IPF. None of these additional ECM proteins were able to successfully predict which patients with IPF were at greatest risk of lung function decline within one year of blood draw.

Published evidence suggests that serum levels of ECM proteins (like collagen) can be reflective of fibrotic changes in the lung (Kasuga et al. 1996), and in this study, the ECM protein periostin, which binds collagen, was increased in the serum of patients with IPF compared to subjects without lung disease. However, prior findings in the literature that reported an increased serum periostin level in patients with IPF who progressed compared to those who remained stable (Naik et al. 2012) were not replicated in this study. One explanation of this discrepancy may be the difference in the blood fraction that was studied.

In this study serum periostin was examined whereas Naik and colleagues investigated plasma periostin levels in patients with IPF. The average plasma periostin level in patients with IPF in the Naik study was approximately 21 µg/mL but the average serum periostin level in patients with IPF in the current study was only 2.9 ng/mL. The main difference between plasma and serum is the lack of clotting factors and cells in the serum (Burnouf et al. 2013) and therefore it is possible that the level of periostin in the serum would be less than in the plasma.

Further support of our initial hypothesis that fibulin-1 is a unique ECM protein biomarker of disease progression in IPF, was drawn from ROC curve analysis, which showed that of the four serum ECM biomarkers tested, only fibulin-1 was able to accurately discriminate between patients with IPF who progressed or those who remained stable. While the 95% confidence intervals for the area under the curve of serum fibulin-1 were wide, they did not cross 0.5. This is an indication that serum fibulin-1 is worthy of further study as a biomarker. If the 95% confidence intervals cross 0.5, then the biomarker has no better than a 50% chance of discriminating between the two cases being compared (in our case whether a patient was likely to progress or remain stable), which was the case for the other three ECM proteins.

The three ECM proteins periostin, tenascin-C and fibronectin have roles in the progression of fibrosis in patients with IPF (Carey et al. 2010; Lepparanta et al. 2012; Naik et al. 2012). Furthermore, increased periostin mRNA has been implicated as a characteristic of myofibroblasts in fibroblastic foci. Although accuracy of disease progression prediction was the characteristic most important in this biomarker study, alterations in ECM levels may still reflect changes in lung function.

When the relationship between lung function and serum ECM proteins in patients across all ILDs was examined, three of the four (all except fibronectin) ECM molecules were increased as FVC% decreased. In addition, both serum fibulin-1 and periostin were increased as $DL_{CO}$% and TLC% decreased, and as CPI increased. Taken together, we begin to illustrate an environment of elevated serum ECM proteins that is reflective of an overall poorer state of lung function and increased disease severity in patients with pulmonary fibrosis.

Lung function decreases as the normal architecture of the lung is replaced by fibrotic tissue. Tissue levels of both fibulin-1 and periostin were increased in patients with IPF compared to subjects without lung disease, but only fibulin-1 correlated with poorer lung function in patients with IPF. It is not hard to imagine why an interlacing, bridging protein like fibulin-1 would be found in greater amounts in the tissue of subjects whose lung function is poor. The physiological effect of increased collagen deposition is not only a consequence of excessive protein production, but also in its changed 3-D structure. A good example of this is the fact that increased collagen deposition is associated with both IPF (McKleroy et al. 2013) and emphysema (Martin-Mosquero et al. 2006), yet the physiological outcome on lung mechanics is drastically different (Faffe and Zin 2009).

The ability of periostin to modulate collagen and fibronectin deposition has been shown in mice (Norris et al. 2007; Kudo 2011) and in this study, we showed that as tissue periostin increases so does tissue collagen and fibronectin in the tissue of patients with IPF. Similarly, the relationship between periostin and tenascin-C (Kii et al. 2010) was reproduced as the proteins correlated in the serum of patients with IPF. The effect size is used to describe the strength of the relationship between two variables and in this case, the relationships between serum fibulin-1 and tenascin-C and between serum tenascin-C and periostin may be considered moderately correlated as the Pearson's correlation coefficients were only 0.4. This effect size may not be accurately reflect the importance of the relationship in biological context as small changes in lung 3D structure may result in a pronounced effect on lung function .

The biomechanics of lung parenchyma is largely determined by the structural arrangement of collagen, elastin and proteoglycans (Suki et al. 2005). The

macromolecules collagen, elastin and fibronectin are capable of forming fibres thicker than several hundred nanometres which form a network that extends from the central airways to the alveolar ducts. The orientation of these large fibres is determined by the smaller interlacing proteins (Culav et al. 1999) and this is the first study to highlight correlations between fibulin-1, periostin, tenascin-C and fibronectin ECM proteins in the serum and tissue of patients with IPF.

While increasing levels of tissue fibulin-1 moderately correlated with increasing levels of periostin, it did not correlate with fibronectin, as we might have expected. This may be due to insufficient power in this study to be able to correctly reject the null hypothesis. However, strong correlations were seen between tissue periostin and fibronectin and between periostin and total collagen as expected from the literature (Hamilton 2008; Kii et al. 2010; Kudo 2011). Interestingly, tissue fibronectin and total collagen did not correlate with each other. This may be explained by the replacement of fibronectin with collagen during fibre maturation as seen in various stages of development (Dessau et al. 1980).

Expression of certain ECM proteins is limited in normal adult tissues, but increased during development/wound healing (Bosman and Stamenkovic 2003) and tenascin-C is one such ECM protein (Imanaka-Yoshida 2012). To our knowledge this is the first study to show that serum tenascin-C has potential as another biomarker in IPF, as it was decreased in the serum of patients with IPF compared to patients with HP, a disease of similar clinical presentation. Conversely, increases in tenascin-C have been shown in the BALF of patients with IPF (Kaarteenaho-Wiik et al. 1998). This may reflect a different source of tenascin-C as both the BALF and route of experimental fibrosis involve the epithelial cells.

However, except for fibulin-1, none of the other three ECM proteins tested were increased in the serum *and* had increased tissue levels that correlated with poorer lung function in patients with IPF. Those two factors (increased serum and tissue levels of the candidate protein) are central in being able to distinguish which patients exhibit an active fibrogenic state and therefore are an indication of the usefulness of that protein as a biomarker.

Activated fibroblasts are known to be the mesenchymal cell most responsible for ECM deposition (Kisseleva and Brenner 2008). Fibroblasts are the key driver in the fibrotic process and the matrix they produce influences those fibrogenic changes (Klingberg et al. 2013). In pulmonary fibrosis, the diseased matrix is drastically altered (Booth et al. 2012; Tschumperlin et al. 2012) and this was reflected in the dysregulation of the ECM proteins in fibroblasts derived from patients with IPF. In this study, basal mRNA expression of tenascin-C is decreased in IPF fibroblasts compared to both Non-IPF and COPD fibroblasts. In contrast, there was no difference in either periostin or fibronectin mRNA levels between patients with IPF and subjects without lung disease and this supports our hypothesis that fibulin-1 is a unique signal of fibrotic fibroblasts.

In the search for a serum biomarker of active fibrogenesis it is important to establish a potential source of the serum protein for future targeted therapies. To complete this investigation, the levels of soluble ECM protein production secreted from the cultures of primary parenchymal fibroblasts were compared between Non-IPF, COPD and IPF fibroblasts. Neither cell-secreted periostin or tenascin-C production was increased in IPF fibroblasts compared to Non-IPF or COPD fibroblasts, results which strengthen

the conclusion that increased fibulin-1 production may be a unique target of activated, and resident, fibrotic fibroblasts.

This study is the first to report that while it is likely that many ECM proteins are dysregulated in the context of idiopathic fibrotic disease, fibulin-1 is a better biomarker than three other ECM proteins that have been identified as participants in the fibrotic process. The variable clinical course in IPF emphasizes the need for serum biomarkers of disease progression that reflect active fibrogenic states in the lung and identify patients who are at a higher risk of lung function decline. In the search for a biomarker, measurements of fibulin-1 may be able to identify patients with IPF who are most likely to rapidly require lung transplantation.

# Chapter 4. **The effect of TGFβ1 stimulation on fibulin-1 production in fibroblasts**

## 4.1 Introduction

An injury to the lung sets off a tightly regulated chain of events that under normal circumstances results in restoration of the lung architecture following closure of the wound. However, in the event of pathological fibrosis, the repair process becomes unrestrained and extracellular matrix (ECM) deposition becomes excessive.

Mesenchymal cells are responsible for the production of most of the ECM and therefore the building blocks of fibrosis. Fibroblasts produce the vast majority of the matrix proteins and provide the basis of the ECM in the interstitium (Frantz et al. 2010). Although pulmonary fibrosis is thought to be driven by an abnormal epithelial repair process that propagates throughout the interstitium (Crosby and Waters 2010), it is the dysregulated ECM production by lung fibroblasts that alters the physical architecture of the tissue and results in the loss of lung function (Faffe and Zin 2009).

Impaired communication between epithelial cells and resident fibroblasts drives aberrant wound repair (Selman and Pardo 2002). Epithelial cells line the airways, cover the alveolar surface and airway epithelial cells can exhibit a thickened basement membrane, which is a specialized ECM, during fibrogenesis. Both epithelial cells and fibroblasts can also produce cytokines that further perpetuate the disease process (Lomas et al. 2012) by recruiting other cells to the injury site. For example, circulating fibrocytes, which are potential progenitors of fibroblasts, are recruited to the lung by chemo-attractant cytokines produced by alveolar epithelial cells and are a

potential source of the increased fibroblast numbers in IPF (Andersson-Sjoland et al. 2008).

The pro-fibrotic cytokine transforming growth factor (TGF)-β1 is a major driver in fibrogenesis and is overproduced in IPF (Lepparanta et al. 2012). TGFβ1 is also important in differentiation of the fibroblast into its pathological and contractile phenotype during pulmonary fibrosis (Kage and Borok 2012), which is characterized by the expression of alpha-smooth muscle actin (α-SMA) (Zhang et al. 1996).

Stimulation of fibroblasts with TGFβ1 induces ECM proteins like fibronectin (Doerner and Zuraw 2009) and type I collagen (Kenyon et al. 2003). TGFβ1 can also bind to other ECM components such as elastin and decorin (Prud'homme 2007), two ECM molecules that affect the overall physiology of the lung (Antunes et al. 2009). TGFβ1 is also known to induce production of periostin (Naik et al. 2012) and tenascin-C (Fitch et al. 2011). Furthermore, latent TGFβ1 co-localizes with fibronectin, indicating that the storage of TGFβ1 and therefore availability are modulated in the fibrotic lung and emphasizing the role of the ECM as an active player in fibrogenesis (Lepparanta et al. 2012).

The ECM protein fibulin-1 is increased following stimulation with TGFβ1 in airway smooth muscle (ASM) cells derived from patients with asthma (Lau et al. 2010). In the asthmatic airway, TGFβ1 is increased and associates with fibroblasts as well as ASM (Howell and McAnulty 2006). As part of the fibrogenic remodelling process in asthma, there is excessive subepithelial deposition of ECM molecules that result in increased basement membrane thickness and, importantly, stiffening of the tracheobronchial walls (Royce et al. 2009).

In the lung, as with other organs, mechanical forces can directly influence cellular behaviour, especially during embryonic development (Kubota et al. 2012). The mechanical properties of the lung parenchyma are in part determined by the structure of the alveolar walls (Suki et al. 2005) and in particular, fibulin-1 is necessary for the embryonic development of the alveolar septa (**Figure 4.1**) (Kostka et al. 2001).

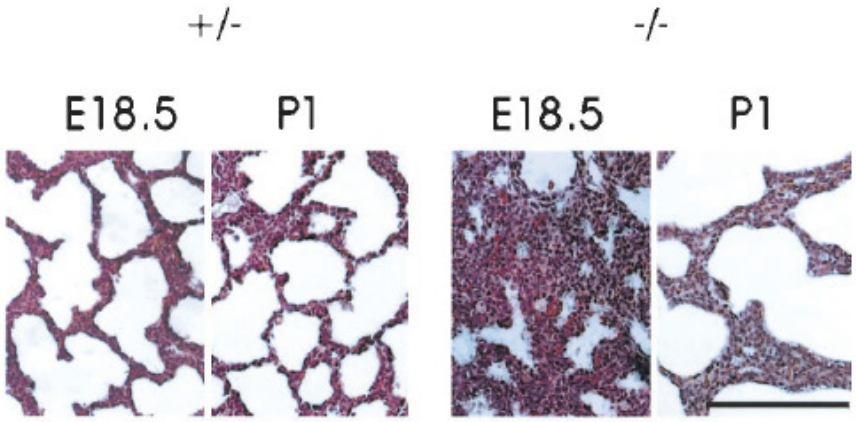



**Figure 4.1 Fibulin-1 is necessary for proper lung development of mice.**
Hemotoxylin (blue, cell nuclei) and eosin (red, collagen/muscle) staining of mouse tissue sections show that mice which are homozygous-deficient for fibulin-1 exhibit improperly expanded sacculi and thickened septa in the lungs at mouse embryonic day 18.5 (E18.5) and one day after birth (P1) compared to heterozygous controls.
Bar 100µm
Adapted with permission from (Kostka et al. 2001).

Fibulin-1 also has an important role in elastic fibre formation (Roark et al. 1995) as it binds to tropoelastin (Sasaki et al. 1999) and several of the central proteoglycans involved in ECM structure such as nidogen (Kubota et al. 2006) and versican (Aspberg et al. 1999), the latter of which is increased in pulmonary fibrosis (Bensadoun et al. 1996). Elastic fibres are also important determinant fibres in the biomechanical properties of the lung and changes in ECM composition in the context of fibrotic disease often includes the dysregulation of elastic fibre molecules (Faffe et al. 2006).

In the lung parenchyma, elastic properties are largely determined by the orientation of elastin fibres and the loosely-arranged collagen fibres that form a spring-like network around cells and vessels (Rocco et al. 2004). Tenascin-C, in concert with periostin, is largely responsible for the proper orientation of collagen fibres (Kii et al. 2010). Fibulin-1 binds fibronectin (Argraves et al. 1989) and is also known to alter the binding of tenascin-C to fibronectin (Williams and Schwarzbauer 2009). The nature of the interconnected relationship between large structural fibres (fibronectin and collagen) and smaller, bridging ECM proteins like fibulin-1 (as well as periostin, and tenascin-C) may be dysregulated within the fibroblasts themselves in the context of fibrotic disease, resulting in an overall stiffer lung because the fibroblasts that populate the ECM are also more stiff. Lung tissue from mice treated with bleomycin (an experimental model of pulmonary fibrosis in which interstitial collagen deposition is induced) is stiffer than lung tissue from saline-treated mice, as measured by atomic force microscopy (AFM) (Liu and Tschumperlin 2011) but to date, there have been no investigations into the stiffness of primary lung fibroblasts derived from patients with and without pulmonary fibrosis.

AFM is a microscopy technique that has been used for decades in material science and has been used on organic materials since the late 1980's (Marti et al. 1988). AFM first characterizes the topography of the material by measuring the deflection of a cantilever attached to a probe as it is passed over the cellular surface (Marti et al. 1988). Secondly, it then estimates the stiffness of the tissue layer by measuring the resistance of the tissue to deformation by the cantilever/probe as it is indented into the tissue (Marti et al. 1988). A stiffer tissue would be more resistant to the pushing of the cantilever (Jalili and Laxminarayana 2004).

Studies with lung fibroblast cell lines have demonstrated that TGFβ1 can increase fibroblast cell stiffness (Liu et al. 2010) and it is likely that a similar relationship can been seen in primary cells. Consequently, we were interested in using AFM to investigate the physical differences between individual primary fibroblast cells as they are grown in culture in both basal and fibrotic conditions using stimulation with TGFβ1.

To this end, in this chapter we aimed to examine the effect of TGFβ1 on fibulin-1 expression and production in fibroblasts derived from patients with IPF, COPD and from subjects without lung disease. Secondly, we aimed to confirm that TGFβ1 also increased the expression of the ECM proteins periostin, tenascin-C and fibronectin. Lastly we examined the morphology and stiffness of fibroblasts in the context of both a basal and TGFβ1-stimulated environment.

## 4.2 Methods

### 4.2.1 Patient Data

The patients from whom the fibroblasts were derived were previously described in Chapter 2 (Table 2.8) and Chapter 3 (Table 3.10).

### 4.2.2 Fibroblast isolation and cell culture

Primary parenchymal fibroblasts were cultured and set up for experiment as previously described in Chapter 2 (Methods 2.2.5, 2.2.6, 2.2.7). For atomic force microscope measurements, cells were washed twice with cold, sterile PBS and fixed for 20 minutes at room temperature with 10% neutral buffered formalin solution (Sigma Aldrich, Sydney, Australia). Cells were washed twice again with PBS as before. Cells were stored in sterile PBS at 4°C until probing.

### 4.2.3 Stimulation with TGFβ1

Activated TGFβ1 (R&D Systems, USA) recombinant protein was used in all experiments. For cell stimulation, TGFβ1 was diluted in quiescing media as described in Chapter 2 (Methods 2.2.7).

### 4.2.4 RNA isolation

RNA isolation was performed as described in Chapter 2 (Methods 2.2.7.1).

### 4.2.5 Real-time reverse transcription polymerase chain reaction

QPCR was performed as described in Chapter 2 (Methods 2.2.8). In addition to the primers that have been described in Chapter 2 (2.2.8.2) and Chapter 3 (Methods 3.2.3), additional primers used in this chapter were for the specific isoforms of

fibulin-1 (A: custom made primer designed by previous PhD candidate Justine Lau ((Lau et al. 2010)), B: Hs00972625_m1, C: Hs00242546_m1, D: Hs0019774_m1) and for IL-6 (Hs00174360_m1), α-SMA (Hs00426835_g1) and Fibulin-5 (Hs00197064_m1).

### 4.2.6 Secreted and cell-associated protein collection from cell cultures

Cell secreted and cell-associated proteins were collected from primary parenchymal fibroblast cultures as described in Chapter 2 (Methods 2.2.7.2).

### 4.2.7 Immunoblotting

Immunoblotting was performed as previously described in Chapter 2 (2.2.9 and 2.2.13) and analysed as described in Chapter 2 (2.2.14).

### 4.2.8 Sandwich ELISA

The levels of periostin and tenascin-C were investigated by sandwich ELISA as previously described in Chapter 3 (3.2.4). Levels of the cytokines IL-6 and IL-8 were measured using antibody pairs from BD (Becton Dickinson & Co, USA), summarised in Table 4.1, using the ELISA method outlined in Chapter 3 (Methods 3.2.4).

**Table 4.1 Materials used in ELISAs for cytokine measurements**

| | IL-6 set (BD cat#55520) | IL-8 set (BD cat#555244) |
|---|---|---|
| Primary antibody | 1µg/mL in 0.1M $Na_2HPO_4$ | 4µg/mL in PBS |
| Blocking media | 1% BSA/PBS | 1% BSA/PBS |
| Standard | 1000pg/mL →0pg/mL | 2000pg/mL → 0pg/mL |
| Samples | Diluted as necessary | Diluted as necessary |
| Secondary antibody | 0.5µg/mL | 40ng/mL |
| Detection antibody | 1:200 (R&D cat#DY998) | 1:200 (R&D cat#DY998) |
| Substrate | TMB (Zymed cat#00-2023) | TMB (Zymed cat#00-2023) |
| Stop solution | 1M $H_3PO_4$ | 1M $H_3PO_4$ |
| Lower limit of detection | 47pg/mL | 31pg/mL |

### 4.2.9 Light microscopy

Images of fibroblasts in culture were taken using an Camedia C4000 Zoom (Olympus, USA) digital camera attached to a CK2 light microscope (Olympus, USA) at 10x magnification.

### 4.2.10 Atomic Force Microscopy

Atomic force microscopy (AFM) was performed under the supervision and tutelage of Dr. Wojciech Chrzanowski (Faculty of Pharmacy, The University of Sydney). The analysis of the results was performed by Jade Jaffar.

For atomic force microscope measurements, cells were washed twice with cold, sterile PBS and fixed for 20 minutes at room temperature with 10% neutral buffered formalin solution (Sigma Aldrich, Sydney, Australia). Cells were washed twice again with PBS as before. Cells were stored in sterile PBS at 4°C until probing.

AFM was performed using an atomic force microscope from Asylum Research (Santa Barbara, CA, USA) with the IGOR Pro software (Version 6.3.4.1, Wavemetrics, Oregon, USA). The cantilever (Asylum Research, Santa Barbara, CA, USA) used was a silicone-nitrate base with a cone-shaped tip. The model used to fit the force curves generated by AFM was the Derjaguin–Muller–Toporov (DMT) model with the invols set to 147nm/V and the spring constant of the cantilever was set to 12.8 piconewtons per nanometer (pN/nm) as per the manufacturer's instructions.

The Poisson ratio of all the samples was set to 0.5. When material is stretched in one direction it will get thinner in the other two directions. The Poisson ratio is a measure of deflection of the sample and relates to the amount of compression that the material can undergo. This compression leads to a larger change in volume in materials with a

higher Poisson's ratio. Most metals have a Poisson's ratio between 0.25 and 0.35 whereas rubber has a Poisson's ratio of 0.5. Biological materials have a Poisson's ratio of 0.5 because the indentation of the cantilever does not produce a permanent change in volume (Buzard 1992). The Poisson's ratio is limited between -1 and 0.5.

Surface topography of fibroblasts was measured in contact mode, where the surface of the sample remains in contact with the tip of the cantilever. The scan rate for topography was 0.2Hz. Cell stiffness of the fibroblasts was measured by contact-indentation mode, where the surface is indented by the cantilever tip and then retracted before the tip moves along to the next measurement point. The scan rate for cell stiffness was also 0.2Hz.

### 4.2.11 Statistical Analysis

Graphs were made using GraphPad Prism 6 Software for Windows (Version 6 GraphPad Software Inc. 1992-2007). Statistical analysis was done using SPSS Statistics (Version 21 IBM Corporation 1989-2012).

The effect of different concentrations of TGFβ1 was assessed using paired t-tests. Differences in the basal levels of ECM proteins between groups of fibroblasts were assessed by one-way ANOVA with Tukey's post test correction for multiple comparisons. Differences between groups before and after stimulation with TGFβ1 were assessed by two-way ANOVA with Tukey's post test correction for multiple comparisons.

## 4.3 Results

The basal levels of fibulin-1, periostin, tenascin-C and fibronectin were reported in the previous Chapters (fibulin-1, Figure 2.12, Figure 2.13; periostin/ tenascin-C/ fibronectin, Figure 3.10, Figure 3.11) and have been reproduced in this Chapter for comparison purposes. Graphical mRNA data is presented using 1/ΔCt levels on the y-axis. Equal amounts of mRNA from each fibroblast line were converted into cDNA and levels of mRNA were quantified by QPCR. The comparative (or ΔΔCt) method was used to calculate the relative abundance of mRNA compared to the housekeeping gene 18S. Samples with a high amount of a particular mRNA transcript have a smaller delta Ct value because fewer cycles of PCR are required to amplify the target gene to a pre-determined threshold value.

### 4.3.1 Characterisation of the basal mRNA expression of fibulin-1 isoforms in primary parenchymal fibroblasts

Alternative splicing of the fibulin-1 gene produces 4 isoforms that differ in their C-terminal region (Tran et al. 1997). Isoforms C and D are the predominant forms expressed in adult human tissues and have been shown to have differing functions during tissue morphogenesis (Muriel et al. 2005). In Chapter 2, this study showed that fibroblasts from patients with IPF produced more fibulin-1 mRNA than fibroblasts from patients without IPF (Figure 2.12) however, whether there was differential expression of the four isoforms of fibulin-1 was not examined. In addition, the levels of fibulin-1 mRNA in fibroblasts derived from patients with COPD was not different compared to fibroblasts derived from patients with and without IPF (Figure 4.2). The basal expression levels of all 4 fibulin-1 isoforms followed the same pattern as shown in Chapter 2 in that fibulin-1A, C, and D were all significantly increased in fibroblasts

from patients with IPF compared to fibroblasts from subjects without lung disease (Two-way ANOVA, $p<0.01$) (Figure 4.2). Fibulin-1B expression was lower than the other fibulin-1 genes and there was no statistical difference in the expression levels in the fibroblasts derived from the three patient groups (patients with IPF, patients without IPF and patients with COPD).

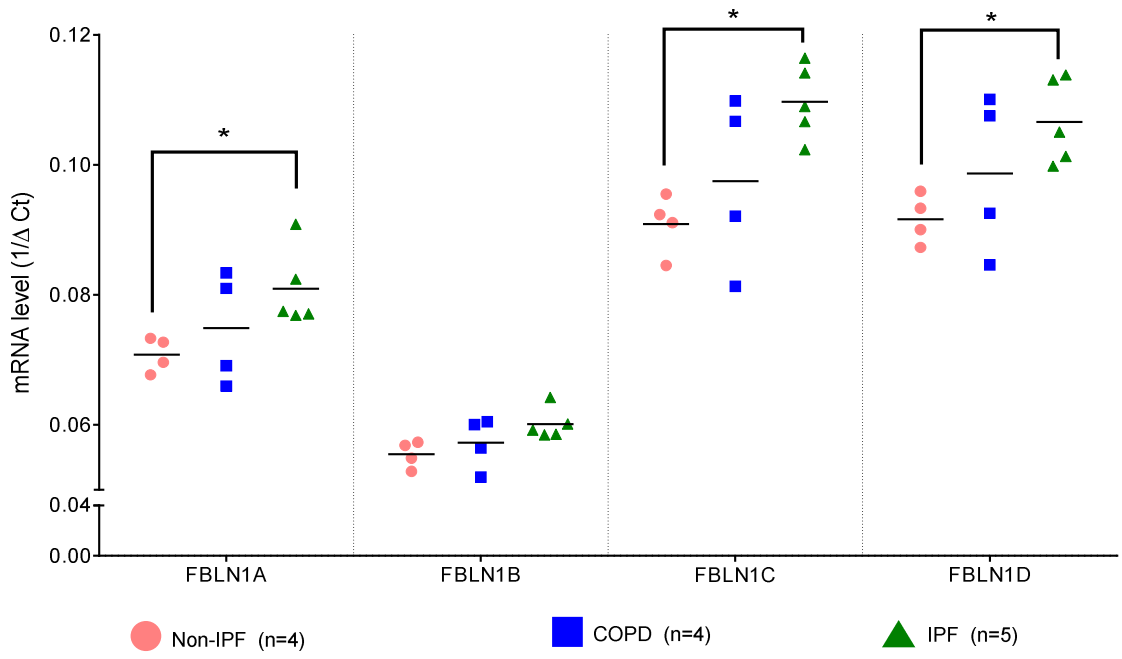



**Figure 4.2 Basal mRNA levels of the four fibulin-1 isoforms in primary parenchymal fibroblasts.**

Primary parenchymal fibroblasts from patients with IPF (n=5) and age and gender matched patients without IPF (n=4) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM, quiesced in 0.1% FBS/DMEM for 24 hours and maintained in fresh 0.1% FBS/DMEM for a further 72 hours. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. 500ng of mRNA was converted to cDNA for this comparison. Data are expressed as 1/delta cycle threshold to 18S (ΔCt) to enable a greater number to reflect more mRNA. The centre line is the population median. *$p<0.05$ one way ANOVA with Tukey's post-test for between group comparisons.
FBLN1 fibulin-1, COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium

Because this study was investigating the effect of TGFβ1 on these fibroblasts, the basal expression of IL-6 and α-SMA, two proteins known to be induced by TGFβ1, was also measured.

In addition, fibulin-5, another member of the fibulin family which also plays a role in elastogenesis (Nakamura et al. 2002) and is also a TGFβ1 gene target (Lee et al. 2008) was measured to elucidate if other fibulin family members were induced by TGFβ1 in primary parenchymal fibroblasts.

There was no difference in basal mRNA expression of IL-6, α-SMA or fibulin-5 between fibroblasts from subjects without IPF and fibroblasts from patients with IPF. Fibroblasts from patients with IPF produced significantly less IL-6 mRNA than fibroblasts from patients with COPD (one-way ANOVA, $p<0.05$) (Figure 4.3).

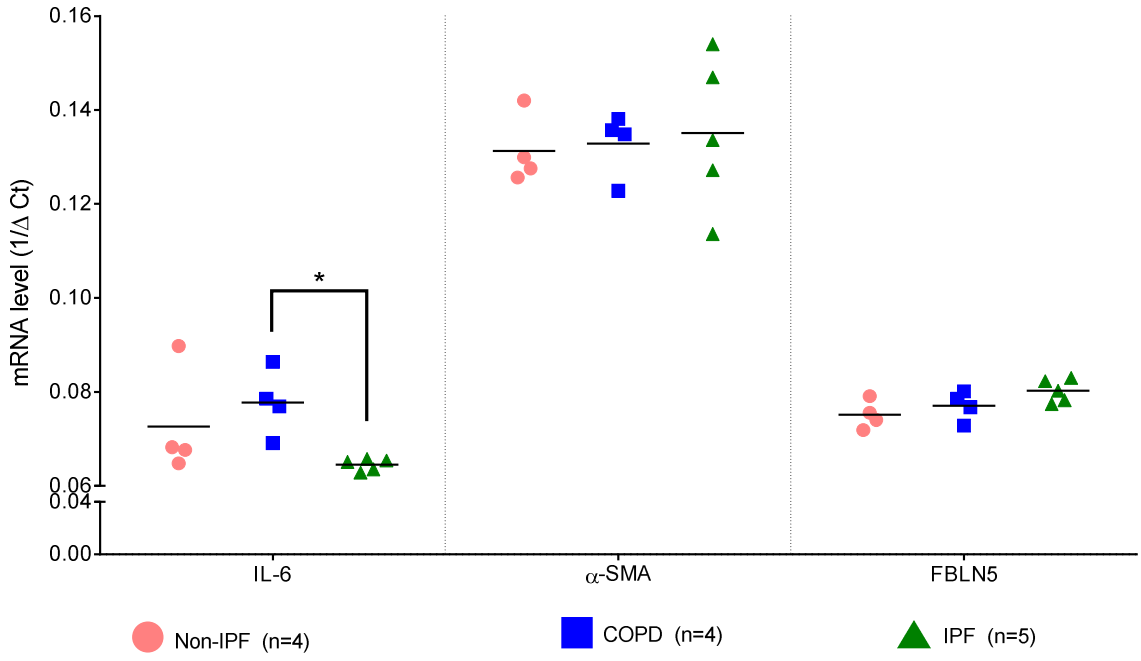



**Figure 4.3 Basal mRNA levels of markers measured to gauge response to TGFβ1 stimulation in primary parenchymal fibroblasts**

Primary parenchymal fibroblasts from patients with IPF (n=5) and age and gender matched patients without IPF (n=4) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and maintained in fresh 0.1% FBS/DMEM/1% pen-strep for a further 72 hours. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. 500ng of mRNA was converted to cDNA for this comparison. Data are expressed as 1/delta cycle threshold to 18S (ΔCt) to enable a greater number to reflect more mRNA. The centre line is the population median. *p<0.05 one way ANOVA with Tukey's post-test for between group comparisons.

FBLN5 fibulin-5, COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, IL interleukin, α-SMA alpha smooth muscle actin, Pen-Strep penicillin-streptomycin

### 4.3.2 The dose related effect of TGFβ1 on fibulin-1 mRNA levels in parenchymal fibroblasts

Initially, the study investigated the effect of different concentrations of TGFβ1 and different times of exposure on fibulin-1C and fibulin-1D mRNA in fibroblasts from 5 subjects without lung disease (Figure 4.4). Fibulin-1C and fibulin-1D were examined as these are the majority isoforms of fibulin-1 in adult humans (Tran et al. 1997). Treatment with three concentrations of TGFβ1 (1, 3, 10ng/mL) significantly downregulated the expression of fibulin-1C and fibulin-1D mRNA at the three timepoints examined, 24 hours, 48 hours and 72 hours of stimulation, with no difference between the time points.

To ensure that the fibroblasts were responding to TGFβ1, the change in fibronectin mRNA was also measured. As expected, TGFβ1 increased fibronectin mRNA at both the 24 hour and 48 hour timepoints, albeit only with the highest concentration of TGFβ1 (Figure 4.5).

For these reasons, further experiments were carried out using 10ng/mL of TGFβ1 for 72 hours.

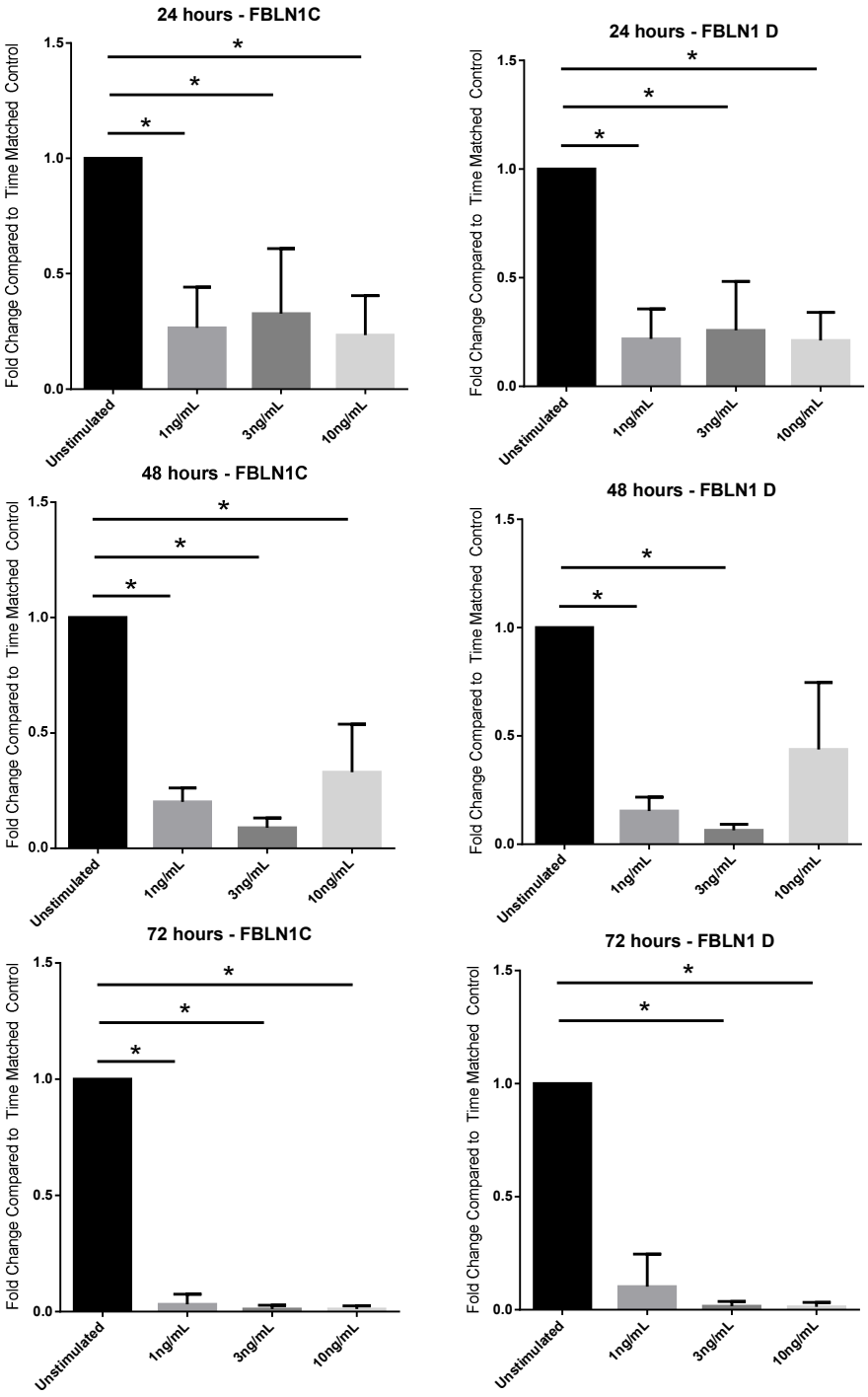



**Figure 4.4 Effect of TGFβ1 on fibulin-1C and fibulin-1D mRNA levels in primary parenchymal fibroblasts from subjects without IPF**

Primary parenchymal fibroblasts from patients without IPF (n=5) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and maintained in fresh 0.1% FBS/DMEM/1% pen-strep or treated with 1, 3, 10ng/mL TGFβ1 in 0.1% FBS/DMEM/1% pen-strep for a further 24, 48 or 72 hours. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. Values are expressed as fold change compared to time matched unstimulated control.

(Paired t-test compared to unstimulated *$p<0.05$).

TGFβ1 transforming growth factor-beta 1, IPF idiopathic pulmonary fibrosis, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

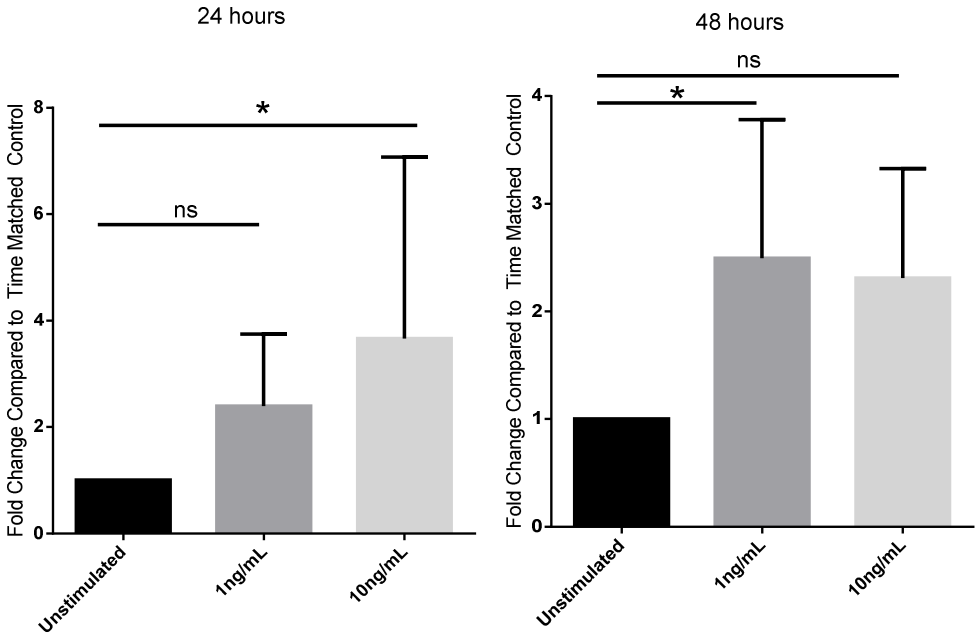



**Figure 4.5 Effect of TGFβ1 on fibronectin mRNA levels in primary parenchymal fibroblasts from subjects without idiopathic pulmonary fibrosis (IPF)**

Primary parenchymal fibroblasts from patients without IPF (n=5) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and maintained in fresh 0.1% FBS/DMEM/1% pen-strep or treated with 1 or 10ng/mL TGFβ1 in 0.1% FBS/DMEM/1% pen-strep for a further 24 (left) or 48 (right) hours. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. Values are expressed as fold change compared to time matched unstimulated control. (Paired t-test compared to unstimulated *$p<0.05$)
TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

### 4.3.3 The effect of TGFβ1 on fibulin-1 mRNA levels of primary parenchymal fibroblasts

Treatment with 10ng/mL of TGFβ1 for 72 hours significantly downregulated all isoforms of fibulin-1, regardless of disease status of the fibroblasts (Figure 4.6).

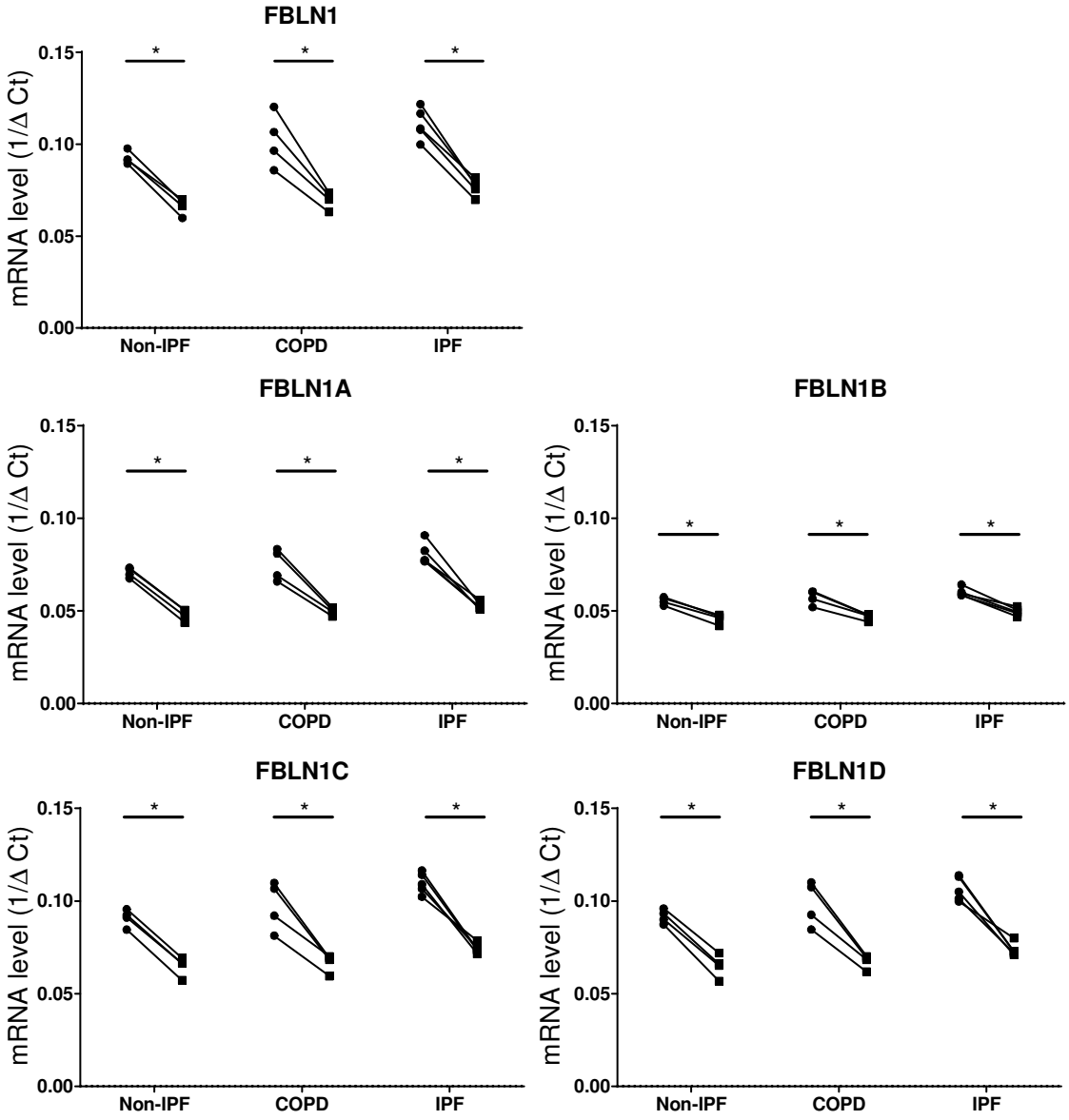



**Figure 4.6 The effect of 10ng/mL TGFβ1 on mRNA levels of fibulin-1 isoforms.**

Primary parenchymal fibroblasts from patients with IPF (n=5) and age and gender matched patients without IPF (n=4) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and either left (●) unstimulated in fresh 0.1% FBS/DMEM/1% pen-strep or (■) stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1% FBS/DMEM/1% pen-strep
(*two-way ANOVA, $p<0.05$)
Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. 500ng of mRNA was converted to cDNA for this comparison. Data are expressed as 1/delta cycle threshold to 18S (ΔCt) to enable a greater number to reflect more mRNA.
COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

### 4.3.4 The effect of TGFβ1 on mRNA levels of other genes of interest in primary parenchymal fibroblasts

Treatment with TGFβ1 significantly increased fibronectin mRNA and α-SMA expression in all 3 disease groups (Figure 4.7). TGFβ1 has been shown to increase α-SMA expression in fibroblasts (Kage and Borok 2012), Treatment with TGFβ1 upregulated periostin mRNA in fibroblasts from patients with IPF ($p<0.05$) but had no effect in fibroblasts from patients without IPF or patients with COPD (Figure 4.7). TGFβ1 stimulation did not alter the mRNA levels of tenascin-C, IL-6 or fibulin-5.

A summary of the means and standard deviations (SD) for the mRNA results is shown in Table 4.2.

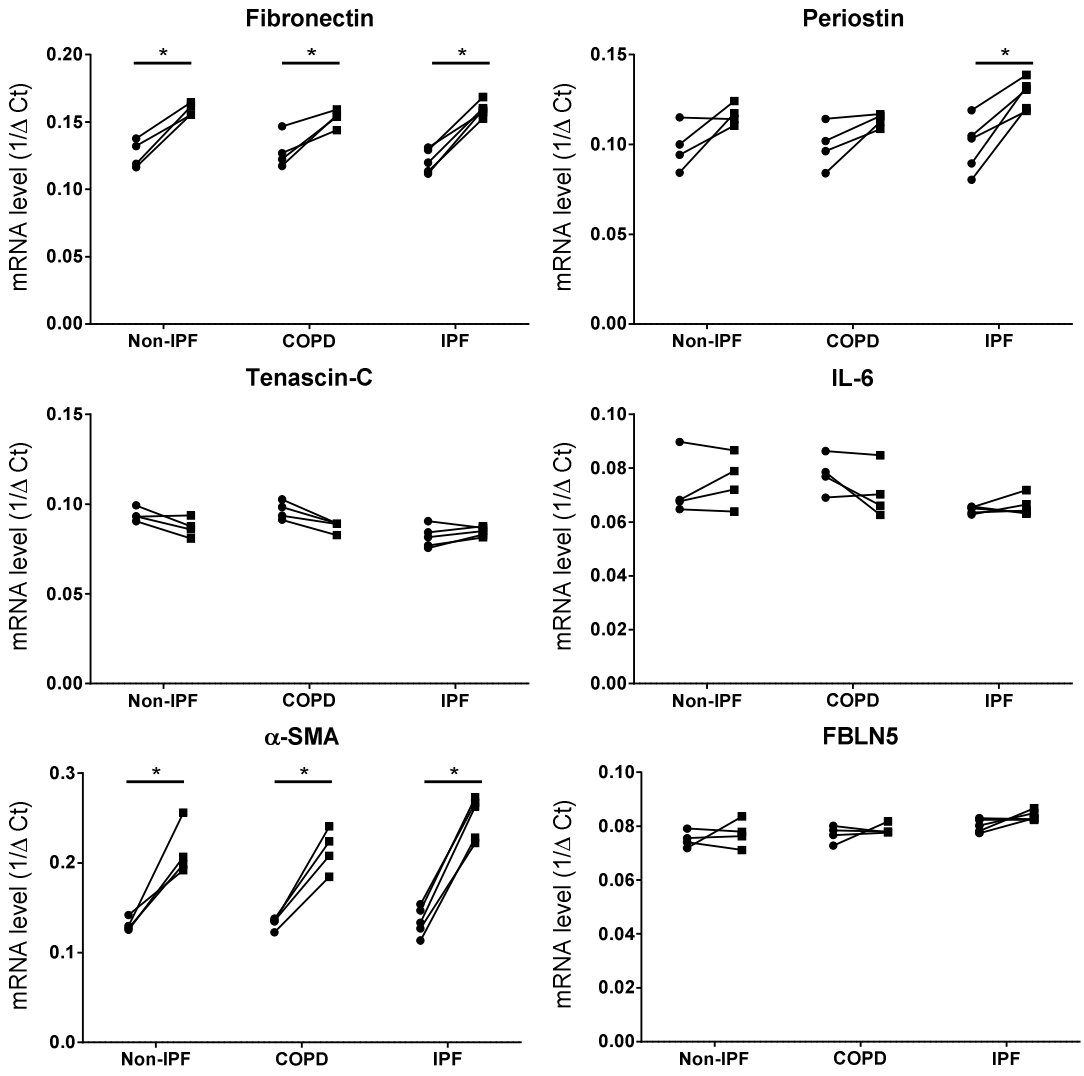



**Figure 4.7 The effect of TGFβ1 on mRNA levels of ECM proteins, IL-6 and α-SMA in fibroblasts.**

Primary parenchymal fibroblasts from patients with IPF (n=5) and age and gender matched patients without IPF (n=4) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and either left (●) unstimulated in fresh 0.1%FBS/DMEM/1% pen-strep or (■) stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1%FBS/DMEM/1% pen-strep (*two-way ANOVA, $p<0.05$). Data are expressed as 1/delta cycle threshold to 18S (ΔCt) to enable a greater number to reflect more mRNA. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. 500ng of mRNA was converted to cDNA for this comparison.
COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin, IL interleukin, SMA smooth muscle actin, FBLN5 fibulin-5

**Table 4.2 Summary of the effect of TGFβ1 on mRNA levels of genes of interest in primary parenchymal fibroblasts**

| mRNA levels (1/ΔCt) | | Non-IPF | | COPD | | IPF | |
|---|---|---|---|---|---|---|---|
| | | Basal | TGFβ1 | Basal | TGFβ1 | Basal | TGFβ1 |
| | (n) | 4 | 4 | 4 | 4 | 5 | 5 |
| FBLN1 (pan) | Mean | 0.093 | 0.066 | 0.102 | 0.070 | 0.111 | 0.077 |
| | SD | 0.004 | 0.004 | 0.015 | 0.005 | 0.009 | 0.004 |
| Periostin | Mean | 0.098 | 0.117 | 0.099 | 0.114 | 0.099 | 0.128 |
| | SD | 0.013 | 0.006 | 0.013 | 0.004 | 0.015 | 0.008 |
| Tenascin-C | Mean | 0.094 | 0.087 | 0.096 | 0.087 | 0.082 | 0.085 |
| | SD | 0.004 | 0.005 | 0.005 | 0.003 | 0.006 | 0.003 |
| Fibronectin | Mean | 0.126 | 0.159 | 0.128 | 0.153 | 0.121 | 0.160 |
| | SD | 0.010 | 0.005 | 0.013 | 0.007 | 0.009 | 0.006 |
| FBLN1A | Mean | 0.071 | 0.048 | 0.075 | 0.050 | 0.081 | 0.053 |
| | SD | 0.003 | 0.003 | 0.009 | 0.002 | 0.006 | 0.002 |
| FBLN1B | Mean | 0.055 | 0.046 | 0.057 | 0.047 | 0.060 | 0.050 |
| | SD | 0.002 | 0.003 | 0.004 | 0.002 | 0.002 | 0.002 |
| FBLN1C | Mean | 0.091 | 0.065 | 0.097 | 0.067 | 0.110 | 0.074 |
| | SD | 0.005 | 0.005 | 0.013 | 0.005 | 0.006 | 0.003 |
| FBLN1D | Mean | 0.092 | 0.065 | 0.099 | 0.067 | 0.107 | 0.074 |
| | SD | 0.004 | 0.006 | 0.012 | 0.004 | 0.007 | 0.004 |
| IL-6 | Mean | 0.073 | 0.075 | 0.078 | 0.071 | 0.065 | 0.066 |
| | SD | 0.012 | 0.010 | 0.007 | 0.010 | 0.001 | 0.004 |
| α-SMA | Mean | 0.131 | 0.213 | 0.133 | 0.214 | 0.135 | 0.251 |
| | SD | 0.007 | 0.029 | 0.007 | 0.024 | 0.016 | 0.024 |
| FBLN5 | Mean | 0.075 | 0.077 | 0.077 | 0.079 | 0.080 | 0.084 |
| | SD | 0.003 | 0.005 | 0.003 | 0.002 | 0.002 | 0.002 |

Primary parenchymal fibroblasts from patients with IPF (n=5) and age and gender matched patients without IPF (n=4) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM /1% pen-strep, quiesced in 0.1% FBS/DMEM /1% pen-strep for 24 hours and either left unstimulated in fresh 0.1% FBS/DMEM /1% pen-strep (Basal) or stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1%FBS/DMEM /1% pen-strep.
Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma. 500ng of mRNA was converted to cDNA for this comparison.
COPD chronic obstructive pulmonary disease , IPF idiopathic pulmonary fibrosis, n Number, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin, IL interleukin, SMA smooth muscle actin, FBLN5 fibulin-5

### 4.3.5 The effect of TGFβ1 on cell-derived ECM proteins in primary parenchymal fibroblasts

The primary focus of this Chapter was the effect of TGFβ1 on cell-derived fibulin-1 because of the hypothesis that increased TGFβ1 may be a driving factor leading to increased fibulin-1 found in the parenchyma tissue, which was shown to correlate with decreased lung function in patients with IPF (Figure 2.11, Table 2.7). Therefore, the effect of TGFβ1 on fibulin-1 protein production in fibroblasts was next examined.

Figure 4.8 shows an example blot of cell supernatants probed for fibulin-1.

Figure 4.9 shows the matching blot of cell lysates from the corresponding samples also probed for fibulin-1.

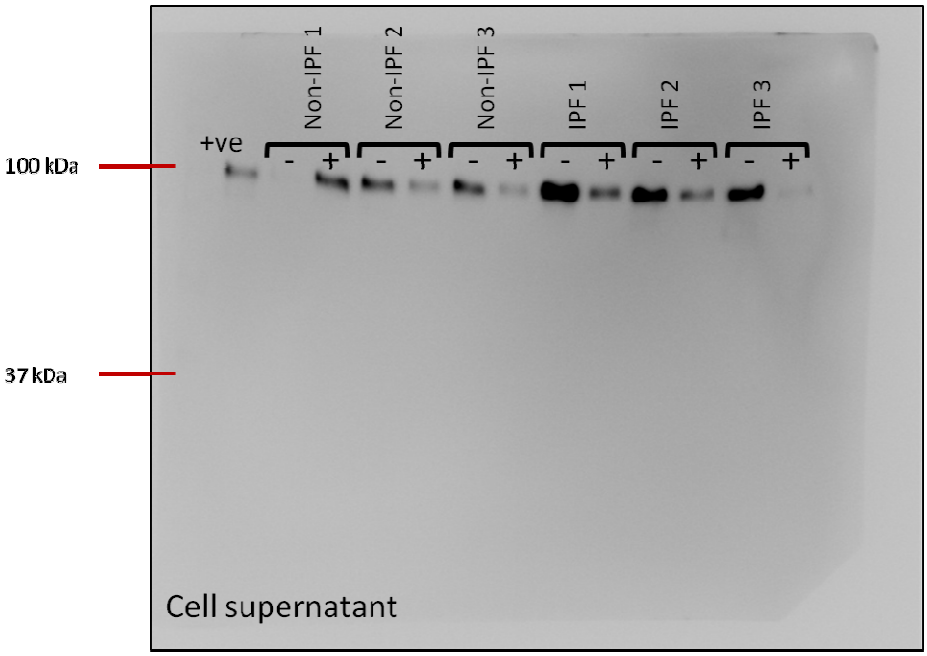



**Figure 4.8 Example blots of supernatants probed for fibulin-1 from fibroblasts derived from patients with and without IPF**

Primary parenchymal fibroblasts from patients with IPF (n=3) and age and gender matched patients without IPF (n=3) were grown for 72 hours in 5% FBS/DMEM /1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep, for 24 hours and either leftunstimulated in 0.1% FBS/DMEM/1% pen-strep (-) or stimulated for a further 72 hours with 10ng/mL TGFβ1in 0.1%FBS/DMEM /1% pen-strep (+).
(+ve) indicates cell secreted fibulin-1 protein as a positive control. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from resections from patients with non-small cell carcinoma.
IPF idiopathic pulmonary fibrosis, n Number, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

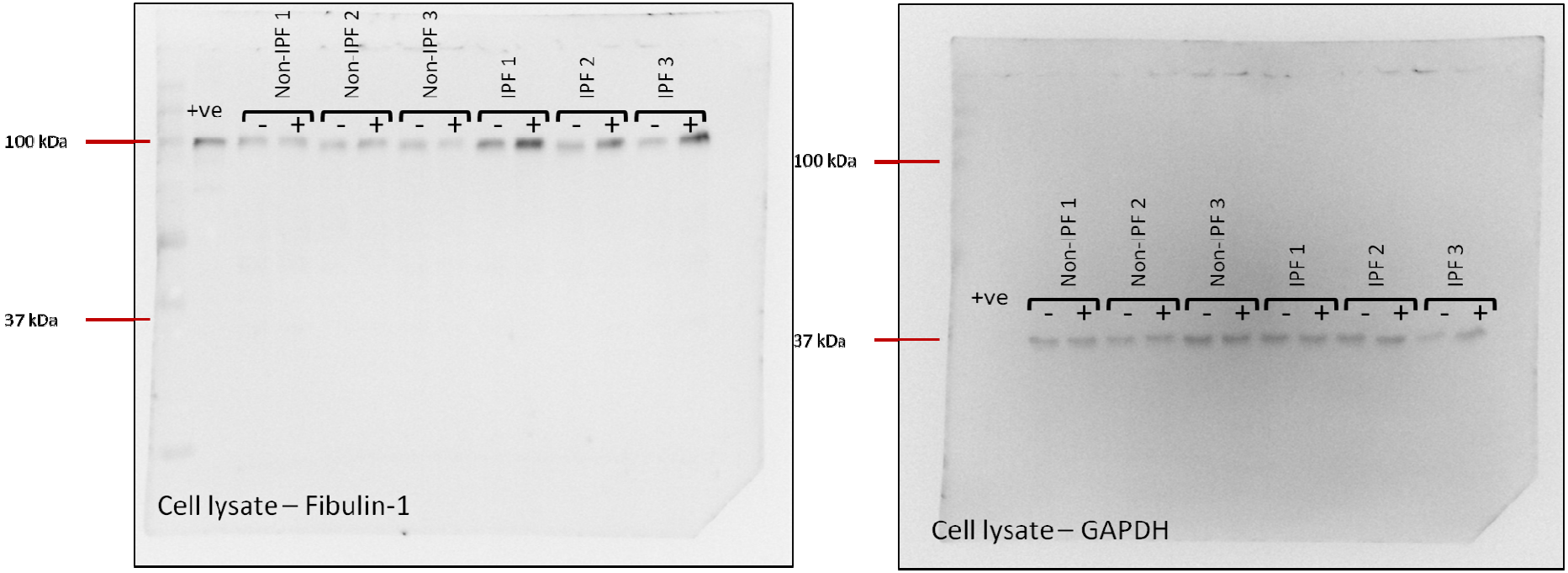



**Figure 4.9 Example blots of cell lysates probed for fibulin-1 and GAPDH from fibroblasts derived from patients with and without IPF**
Primary parenchymal fibroblasts from patients with IPF (n=3) and age and gender matched patients without IPF (n=3) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and either left (-) unstimulated in 0.1% FBS/DMEM/1% pen-strep or (+) stimulated for a further 72 hours with 10ng/mL TGFβ1in 0.1%FBS/DMEM/1% pen-strep. (+ve) indicates cell secreted fibulin-1 protein as a positive control.
Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from resections from patients with non-small cell carcinoma. IPF idiopathic pulmonary fibrosis, n Number, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin, GAPDH Glyceraldehyde 3-phosphate dehydrogenase

There was a differential effect of TGFβ1 on the cell-secreted ECM proteins that were measured. Treatment with TGFβ1 significantly decreased fibulin-1 levels and this effect was consistent across all the disease groups. Conversely, TGFβ1 increased periostin levels in fibroblasts from patients with IPF, but had no effect on periostin levels in fibroblasts from subjects without IPF or with COPD (Figure 4.10).

TGFβ1 significantly increased the production of IL-6 in both fibroblasts from subjects without lung disease and from patients with IPF (Figure 4.11A) however, there was no significant effect on fibroblasts from patients with COPD.

In the absence of stimulation, fibroblasts from patients with COPD produced a significantly higher level of both IL-6 and IL-8 cytokines compared to fibroblasts from subjects without IPF and from patients with IPF (Figure 4.11). Increased levels of IL-6 and IL-8 have been reported in fibroblasts derived from patients with COPD (Zhang et al. 2012). There was no effect of TGFβ1 on IL-8 production in any of the three disease groups (Figure 4.11B).

4.3.5.1 The effect of TGFβ1 in cell-secreted protein production of ECM proteins.

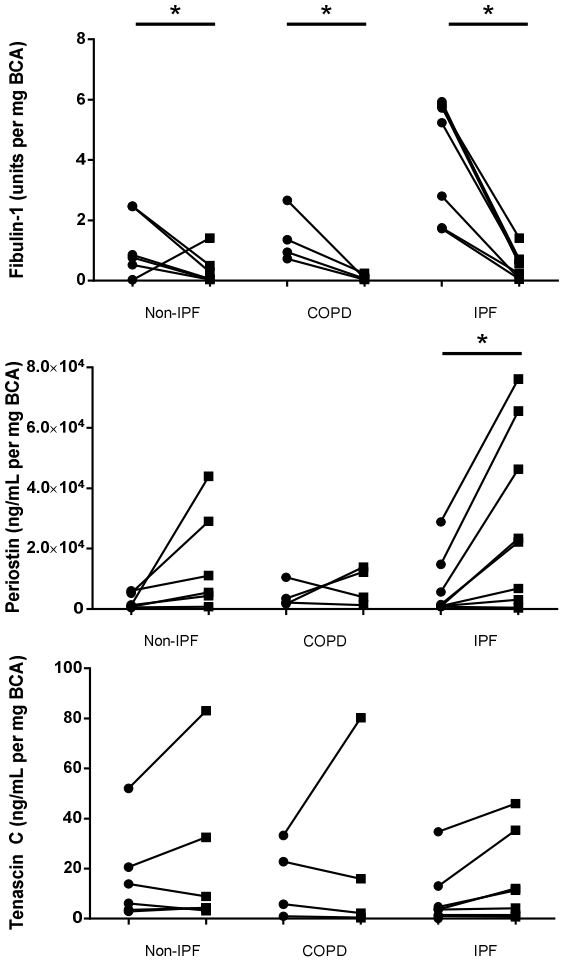



**Figure 4.10 The effect of TGFβ1 on cell-secreted ECM protein production in primary parenchymal fibroblasts.**

Primary parenchymal fibroblasts from patients with IPF (n=8) and age and gender matched patients without IPF (n=7) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS DMEM/1% pen-strep for 24 hours and either left unstimulated in fresh 0.1% FBS/ DMEM/1% pen-strep (●) or stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1% FBS/ DMEM/1% pen-strep (■) (*paired t-test, $p<0.05$).
Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma..
Values were normalized to the total protein content of the cell monolayer as measured by BCA assay.
BCA Bicinchroninic Acid, ECM extracellular matrix, COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

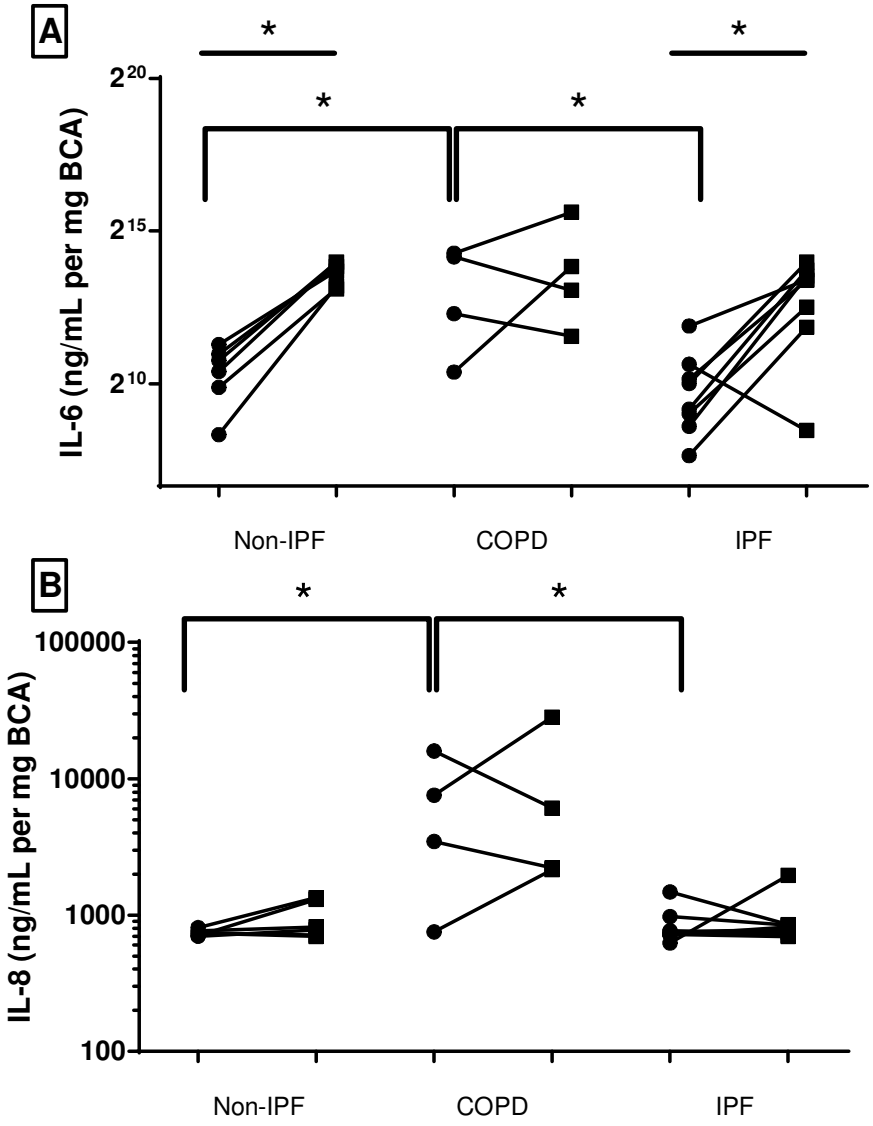



**Figure 4.11 The effect of TGFβ1 on cell-secreted cytokine protein production in primary parenchymal fibroblasts.**

Primary parenchymal fibroblasts from patients with IPF (n=8) and age and gender matched patients without IPF (n=7) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM, quiesced in 0.1% FBS/DMEM for 24 hours and either left (●) unstimulated or (■) stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1% FBS/DMEM (*paired t-test, $p<0.05$).
(A) IL-6 and (b) IL-8 in cell culture supernatants were measured by ELISA. Values (ng/mL) were normalized to the total protein content of the cell monolayer as measured by BCA assay.
Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma.
BCA Bicinchroninic Acid, COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium,

### 4.3.6 The effect of TGFβ1 on cell-associated fibulin-1 production by primary parenchymal fibroblasts

Basal expression of cell-associated (containing intracellular and membrane bound proteins) fibulin-1 protein was increased in fibroblasts from patients with IPF (Chapter 2, Figure 2.8) compared to fibroblasts from patients without IPF and patients with COPD. Therefore the effect of TGFβ1was investigated in this system. Treatment with TGFβ1 significantly increased cell-associated fibulin-1 production by fibroblasts derived from patients with IPF (Figure 4.12). There was no effect of TGFβ1 in either of the other two groups. **Figure 4.9** shows a representative western blot.

### 4.3.7 Basal morphology of primary parenchymal fibroblasts

The basal morphology of fibroblasts from patients with and without IPF was then qualitatively examined. Cultures of fibroblasts derived from three subjects without lung disease and three patients with IPF were used to generate data addressing the final aim of this Chapter. Cells were grown on uncoated plastic dishes. There were no macroscopic differences in gross morphology between the cultures of fibroblasts grown in basal conditions (Figure 4.13).

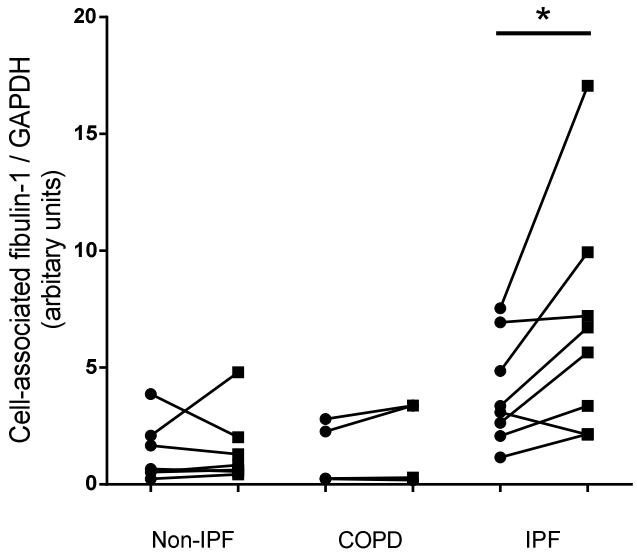



**Figure 4.12 The effect of TGFβ1 on cell-associated fibulin-1 production by primary parenchymal fibroblasts.**

Primary parenchymal fibroblasts from patients with IPF (n=8) and age and gender matched patients without IPF (Non-IPF, n=7) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS DMEM/1% pen-strep for 24 hours and either left unstimulated in fresh 0.1% FBS/ DMEM/1% pen-strep (●) or stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1% FBS/ DMEM/1% pen-strep (■). Cell lysates were collected and analysed by western blot. Blots were stripped and reprobed for GAPDH as loading control (*paired t-test, $p<0.05$) and data were normalised to these protein levels.
Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma.
COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, GAPDH Glyceraldehyde 3-phosphate dehydrogenase, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium , Pen-Strep penicillin-streptomycin

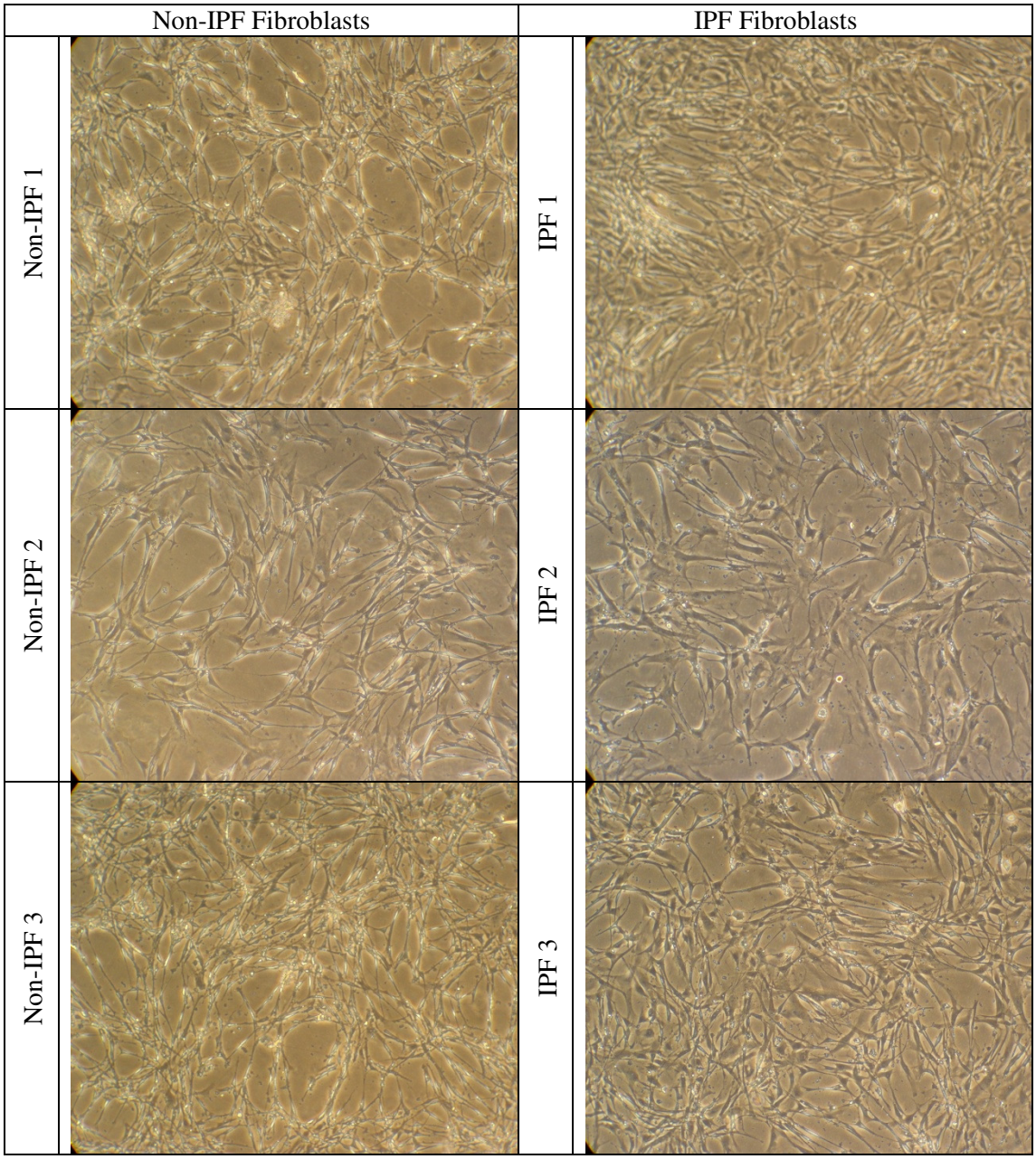
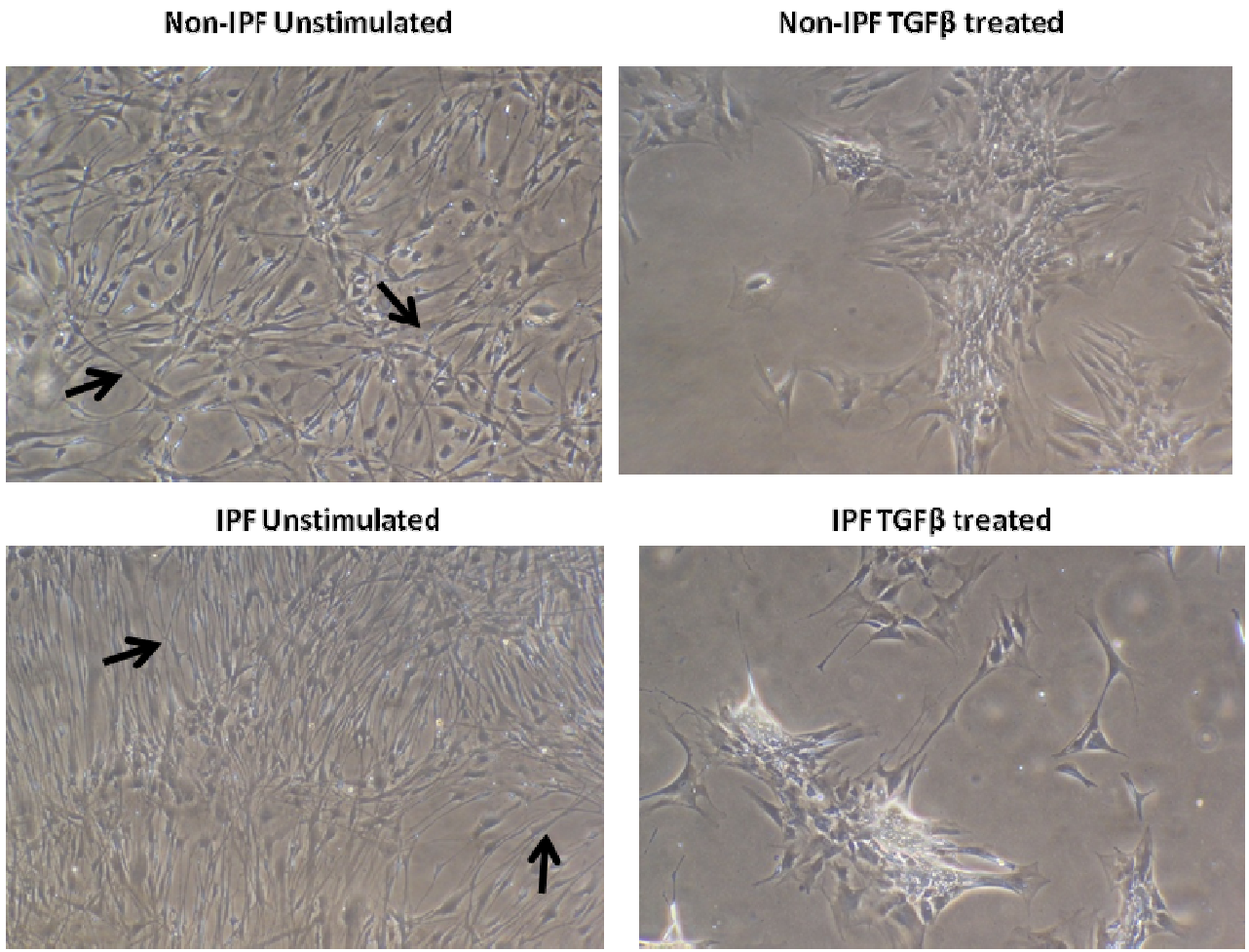



**Figure 4.13 Images of fibroblast monolayers from patients with and without IPF.** Primary parenchymal fibroblasts from patients with IPF (n=3) and age and gender matched patients without IPF (n=3). Cells were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and maintained in fresh 0.1% FBS/DMEM/1% pen-strep for a further 72 hours. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma.
IPF idiopathic pulmonary fibrosis, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin
Magnification 10x

### 4.3.8 The effect of TGFβ1 on cell morphology

Qualitative changes in morphology were seen in all fibroblast cultures when treated with 10ng/mL TGFβ1 for 72 hours (Figure 4.14). Regardless of disease status, fibroblasts responded to TGFβ1 treatment by assembling in tight colonies and losing their spindle-like extensions that are seen under basal conditions.

Next, the effect of treatment with TGFβ1 on the surface topology and stiffness of the cells was quantified by AFM. Scans were performed on two cell lines, one from a patient with IPF and one from a patient without IPF. Each cell line was either treated with TGFβ1 or left unstimulated and a single area, measuring 50µm by 50µm, from each sample was investigated. Areas of similar gross apperance were selected to undergo AFM scanning. The surface topography of the four samples is shown in Figure 4.15.




Magnification 10x

**Figure 4.14 Fibroblasts treated with TGFβ1 undergo morphological changes.**

Representative light microscopy images of primary parenchymal fibroblasts from patients without (top panels) or with idiopathic pulmonary fibrosis (IPF) (bottom panels) are shown. Cells were grown for 72 hours in 5% FBS/DMEM/ 1% pen-strep, quiesced in 0.1% FBS/ DMEM/1% pen-strep for 24 hours and either left unstimulated in fresh in 0.1% FBS/DMEM/1% pen-strep or stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1% FBS/DMEM /1% pen-strep. Images are representative of three fibroblast lines per group.
Arrows indicate places of spindle-like extensions of fibroblasts. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma.
TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin.

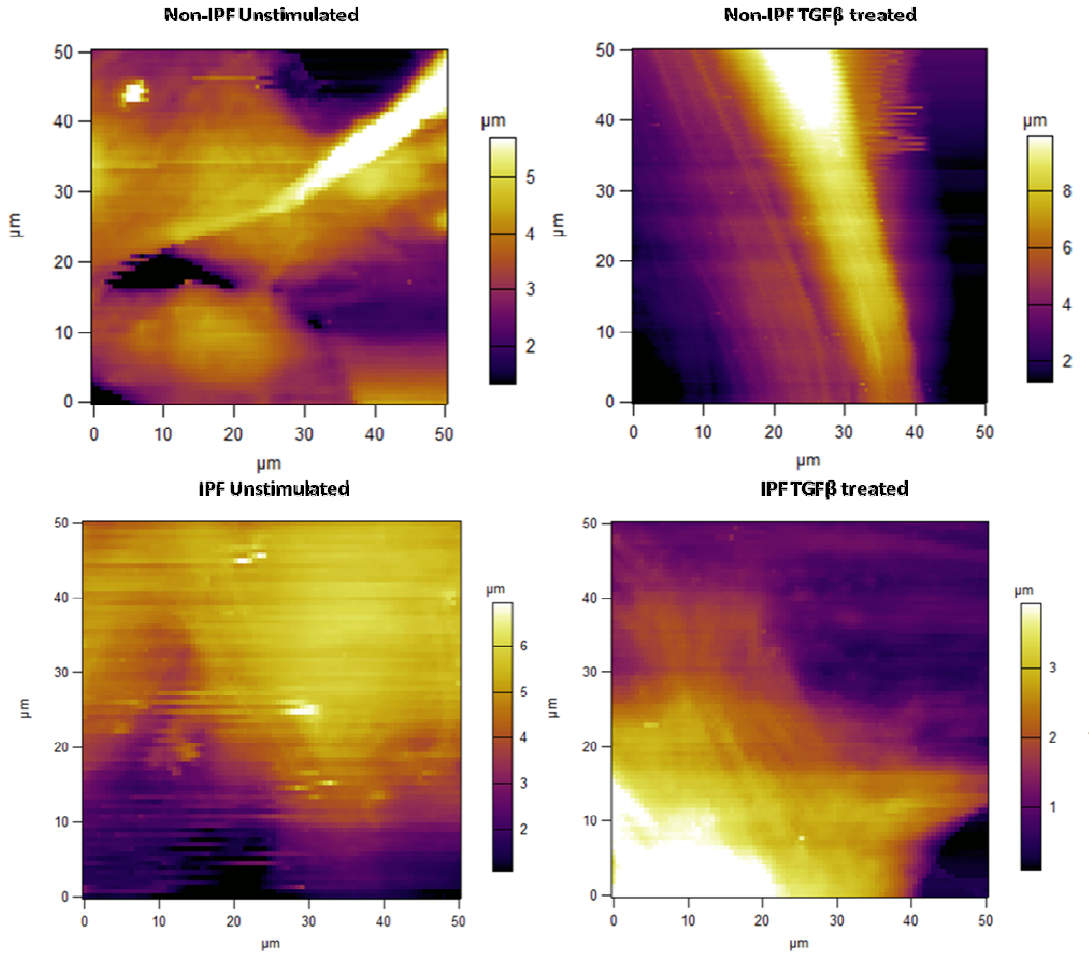



**Figure 4.15 Surface morphology of fibroblasts derived from patients with and without idiopathic pulmonary fibrosis (IPF) as measured by atomic force microscopy.**

Cells were grown for 72 hours in 5% FBS/DMEM /1% pen-strep, quiesced in 0.1% FBS/DMEM /1% pen-strep for 24 hours and either left unstimulated in fresh 0.1% FBS/DMEM /1% pen-strep or stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1% FBS/DMEM /1% pen-strep.
Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from a patient with non-small cell carcinoma.
Images are representative of 1-3 scans per patient.
TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

After scanning for surface topology, the same sample area was then probed for cell stiffness. A representative scan from one of the four samples is shown in Figure 4.16.

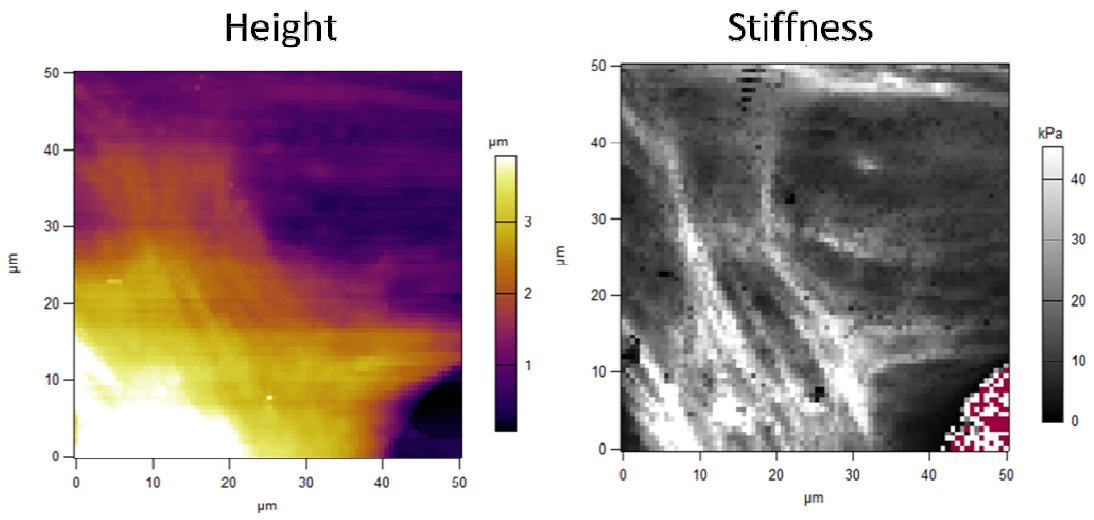



**Figure 4.16 Representative cell height and stiffness scan of fibroblasts as measured by atomic force microscopy.**
Fibroblasts were grown on plastic dishes and first scanned for surface topography. The "height" image was generated by measuring the deflection of the cantilever from a predetermined set point as the cantilever was moved across the surface. Subsequently, the same area was probed for "stiffness" by measuring the deflection of the same cantilever as the cantilever was indented into the cell surface. Points of red indicate where there was no measurement due to instrumental error.

These data suggest that there may be a difference in the response to TGFβ1 between cells derived from patients with IPF compared to cells derived from patients without IPF. The summary of the force measurements of fibroblast stiffness is found in Table 4.3.

The maximum stiffness of fibrotic lung tissue has been reported to be approximately 50kPa (Liu and Tschumperlin 2011) but this figure can change depending on which model is used to calculate the stiffness from the forces measured using AFM. Furthermore, where the cells are of minimal thickness, the influence of the underlying tissue culture plastic may skew the data. Tissue culture plastic has a stiffness of gigapascals (Wells 2008). Therefore the median and interquartile range (IR) of stiffness measurements were initially used to compare the samples.

Unstimulated cells from a patient without IPF had a median stiffness of 0.6kPa (IR 0.2 – 1.1) whereas unstimulated cells from a patient with IPF had a median stiffness of 0.8kPa (IR 0.5 – 1.2). Under stimulation with TGFβ1, cells from a patient without IPF increased in median stiffness to 3.0kPa (IR 1.8 to 6.9), which was an increase of 400%. Cells from a patient with IPF also increased median stiffness under stimulation with TGFβ1to 15.3kPa (IR 10.8 to 24.7), which was an increase of over 1700%.

**Table 4.3 Summary of Force Measurements on human lung fibroblasts**

| | Non-IPF unstimulated | Non-IPF TGFβ1 | IPF unstimulated | IPF TGFβ1 |
|---|---|---|---|---|
| Number of points measured* | 6400 | 6368 | 6400 | 6338 |
| Number of points not analysed** | 0 | 16 | 0 | 62 |
| Average stiffness (kPa) | 4.97 | 26.45 | 0.99 | 22.53 |
| Standard Deviation | 57.34 | 72.47 | 3.55 | 40.55 |
| Minimum Stiffness (kPa) | 0.005 | 0.021 | 0.046 | 0.169 |
| Maximum Stiffness (kPa) | 3460.4 | 514.6 | 158.7 | 700.1 |
| Median Stiffness (kPa) | 0.551 | 3.046 | 0.839 | 15.349 |
| $25^{th}$ Percentile (kPa) | 0.249 | 1.846 | 0.475 | 10.841 |
| $75^{th}$ Percentile (kPa) | 1.128 | 6.855 | 1.184 | 24.737 |

*Number of points measured in a 50 x 50µm area
**Unable to analyse due to instrument error
Primary parenchymal fibroblasts were grown for 72 hours in 5% FBS/DMEM /1% pen-strep, quiesced in 0.1% FBS/DMEM /1% pen-strep for 24 hours and either left unstimulated in fresh 0.1% FBS/DMEM /1% pen-strep or stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1% FBS/DMEM /1% pen-strep. Surface stiffness of cell cultures was measured using atomic force microscopy (AFM) and images were generated using Asylum Research software (Igor Pro Version 6.34A). Stiffness (Young's modulus) was calculated using the Derjaguin–Muller–Toporov (DMT) model. Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from a patient with non-small cell carcinoma.
Data are generated from a single scan on each cell line per treatment.
IPF idiopathic pulmonary fibrosis, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

The change in the proportions of stiffness measurements that fell between 0 and 50kPa in the two different cell lines was examined. For this study, a measurement of 0 to 1kPa was considered normal whereas a measurement of greater than 1kPa to less than or equal to50kPa was considered fibrotic. This range (0 to 50kPa) was chosen to encapsulate the biological range of stiffness measurements and to discount any potential influence of the underlying tissue culture plastic, which measures approximately $10^6$kPa (Discher et al. 2005). This assumption was necessary as the maximum measured stiffness in the current experiments was 3460kPa. In all cases, the total number of points that was disregarded (eg. points that measured more than 50kPa) was less than 10% of the total number of points that were measured. This indicates that the vast majority of each sample area contained cellular material and not plastic.

Because the total number of points that was successfully measured differed and the influence of tissue culture plastic may also have differed between the samples the data was then expressed as percentages of the "Usable Total". The Usable Total was defined as the total number of points that measured less than or equal to 50kPa. The data were normalised by dividing the number of points that fell into each of six consecutive and non-overlapping intervals of stiffness (bins) by the Usable Total. This gave the relative frequencies, or the proportion of cases that fell into each of the bins.

The summary of the relative frequencies is found in Table 4.4. Untreated fibroblasts from a patient with IPF exhibited more stiffness values in the fibrotic range than untreated fibroblasts from a patient without IPF. Fibroblasts from a patient with IPF cultured in non-stimulating conditions had 38% of points of stiffness measure over

1kPa whereas fibroblasts from a patient without IPF grown in the same manner had only 27% of points of stiffness measure over 1kPa.

Treatment with TGFβ1 had a similar effect on fibroblasts from patients with and without IPF. When stimulated with TGFβ1, fibroblasts from a patient without IPF increased to 94% fibrotic (defined as points of stiffness greater than 1kPa) while fibroblasts from a patient with IPF were 100% fibrotic. In both groups of fibroblasts, cells that were treated with TGFβ1 increased the proportion of points that were in the higher stiffness bins. For example, the proportion of points that was greater than 10kPa but less than or equal to 50kPa was 3% and 0% in unstimulated cells from a patient without IPF and from a patient with IPF respectively. Treatment with TGFβ1 increased the proportion of points in that category to 10% and 80% respectively.

**Table 4.4 Treatment with TGFβ1 increases the relative frequency of points in the categories of higher stiffness in lung fibroblasts.**

| | Non-IPF unstimulated | | Non-IPF TGFβ1 | | IPF unstimulated | | IPF TGFβ1 | |
|---|---|---|---|---|---|---|---|---|
| Stiffness bin (kPa) | Count* | Relative Frequency** | Count* | Relative Frequency** | Count* | Relative Frequency** | Count* | Relative Frequency** |
| $0 > x \leq 0.2$ | 1253 | 20% | 7 | 0.12% | 276 | 4% | 1 | 0.02% |
| $0.2 > x \leq 0.5$ | 1714 | 27% | 65 | 1% | 1406 | 22% | 2 | 0.03% |
| $0.5 > x \leq 1$ | 1657 | 26% | 259 | 5% | 2280 | 36% | 7 | 0.11% |
| $1 > x \leq 5$ | 604 | 10% | 1506 | 27% | 2294 | 36% | 36 | 1% |
| $5 > x \leq 10$ | 882 | 14% | 3232 | 57% | 135 | 2% | 1171 | 19% |
| $10 > x \leq 50$ | 177 | 3% | 575 | 10% | 3 | 0.00047% | 4937 | 80% |
| More than 50kPa | 113 | | 581 | | 6 | | 184 | |
| Total points measured | 6400 | % of total points measured | 6224 | % of total points measured | 6400 | % of total points measured | 6338 | % of total points measured |
| Usable Total*** | 6287 | 98% | 5643 | 91% | 6394 | 100% | 6154 | 97% |

*Count indicates the number of points (x) that fell into each stiffness bin
**Relative Frequency is the count of each stiffness bin divided by the Usable Total
***The Usable Total is the number of points measured that fell between 0 and 50kPa.
TGFβ1 transforming growth factor-beta 1, IPF idiopathic pulmonary fibrosis, kPa kilopascal

## 4.4 Discussion

The aim of this chapter was primarily to investigate if fibulin-1 levels in primary parenchymal fibroblasts were altered by treatment with TGFβ1, a pro-fibrotic pluripotent cytokine and a key driver of the fibrotic process. Previous studies had indicated that TGFβ1 had the ability to induce fibulin-1 production in the context of fibrotic airways disease (Lau et al. 2010) and therefore possibly played a role in interstitial fibrosis as many of the mechanisms driving fibrogenesis in lung diseases are shared (Hardie et al. 2009). Therefore, this study aimed to investigate the effect of TGFβ1 on lung fibroblast-derived fibulin-1 and the ECM molecules (periostin, tenascin-C and fibronectin) investigated in earlier chapters as TGFβ1 is known to induce these proteins in other mesenchymal cells (Horiuchi et al. 1999; Doerner and Zuraw 2009). Lung fibroblasts, regardless of disease status, change morphologically when treated with TGFβ1, and these results in turn suggest may result in increased cell stiffness. A summary of the results in Chapter 4 is found in

Table **4.5**.

This study provides evidence that stimulation with 10ng/mL TGFβ1 significantly decreases fibulin-1 mRNA at 24, 48 and 72 hours in fibroblasts derived from patients without IPF (Non-IPF fibroblasts), patients with emphysema (COPD fibroblasts), or patients with IPF (IPF fibroblasts). This decline in fibulin-1 mRNA levels across all disease groups was consistent and the mRNA levels for all four isoforms of fibulin-1 were reduced by the TGFβ1 treatment. This shows that although there were differences in basal levels of fibulin-1 isoforms between disease groups, these differences are negated in the presence of TGFβ1, resulting in similar levels of fibulin-1 mRNA in fibroblasts regardless of disease group. The depressive effect of TGFβ1 treatment on fibulin-1 mRNA is also seen in airway smooth muscle (ASM) cells derived from patients with and without COPD (Chen et al. 2013), indicating that this phenomenon is not limited to parenchymal fibroblasts.

In contrast, treatment with TGFβ1 had a consistent inductive effect on both fibronectin and α-SMA mRNA across the board, a finding which is supported by previous reports in the literature (Thannickal et al. 2003; Dunkern et al. 2007) and indicates a successful response to TGFβ1 stimulation in our study. The effect of TGFβ1 on cytokine production in fibroblasts was also supported by the literature (Ge et al. 2012). Finally, in the absence of stimulation, COPD fibroblasts produced a significantly higher level of both IL-6 and IL-8 cytokines compared to Non-IPF and IPF fibroblasts, again consistent with previous reports in the literature (Zhang et al. 2012). These data provide evidence that the differences in fibulin-1 production among fibroblasts from patients with different diseases are disease specific.

Messenger RNA and protein levels do not necessarily track with one another (Kalinichenko et al. 2008). As this study was investigating the fibrotic signal found in the circulation, the effect of TGFβ1 treatment on the soluble, cell-secreted form of the ECM molecules was examined. Differences in soluble protein regulation between the disease types examined were identified, consistent with earlier literature investigating tenascin-C and periostin (Carey et al. 2010; Yamaguchi et al. 2013).

This is the first study to show that TGFβ1 significantly decreased the amount of cell-secreted fibulin-1 produced by fibroblasts in all the disease groups studied. In addition, a concomitant increase in cell-associated fibulin-1 levels was observed only in IPF fibroblasts. The simplest explanation for this is that TGFβ1 directly drives cell-secreted fibulin-1 into the ECM although the source of the soluble fibulin-1 may in fact be coming from the cell culture media. However, in previous studies from our laboratory, treatment of ASM cells with TGFβ1 also decreased cell-secreted fibulin-1 levels derived from the cells while increasing cell-associated fibulin-1. Importantly, the subsequent increase in cell-associated fibulin-1 levels were not due to *de novo* synthesis of protein, but rather from requisition of cell-secreted fibulin-1 (Chen et al. 2013).

Many ECM proteins are likely to be dysregulated during fibrosis, therefore, due to the promiscuous nature of TGFβ1, this study investigated the effect on ECM proteins which were potential candidate biomarkers of disease progression in IPF. Periostin mRNA has been reported to be increased in IPF fibroblasts compared to Non-IPF fibroblasts (Naik et al. 2012). This finding was not confirmed in this study, albeit this may be explained by a difference in experimental timings between the studies. However, following reports in periodontal ligament fibroblasts (Wen et al. 2010), and

embryonic lung fibroblasts (Takayama et al. 2006), this study confirmed that TGFβ1 increased periostin mRNA in lung IPF fibroblasts. Furthermore, this is the first study to report increases in periostin *protein* following TGFβ1 stimulation in adult lung fibroblasts, although TGFβ1 has been shown to induce cell-secreted periostin protein in transfected bone stromal cells previously (Oku et al. 2008).

TGFβ1 did not upregulate either cell-secreted periostin or tenascin-C protein in Non-IPF or COPD fibroblasts. However, in IPF fibroblasts, cell-secreted periostin but not tenascin-C was increased. In the literature, cell-associated periostin increases with TGFβ1 treatment in mouse osteoblast cells (Horiuchi et al. 1999), supporting the data generated in this study. Periostin has been identified as a potential biomarker of disease progression in IPF (Naik et al. 2012) and its significance as a protein that stabilises the ECM is shown through its interactions with tenascin-C and collagen (Kii et al. 2010). The current study indicates that periostin may also be an important contributor to the fibrotic process in the presence of increased levels of TGFβ1.

Periostin, which is produced as a cytokine from airway epithelial cells (Sidhu et al. 2010), can induce a TGFβ-dependent secretion of type I collagen by airway fibroblasts. Periostin is known to stimulate the TGFβ pathway itself (Sidhu et al. 2010). It is possible that a similar mechanism of ECM modulation is at work in the distal parenchymal fibroblasts as well. In solution, periostin mixes with collagen and alters its cross-linking or fibrillogenesis (Sidhu et al. 2010) and may explain the increased lung tissue stiffness seen in fibrosis (Norris et al. 2007). This action of soluble ECM molecules affecting the 3-dimensional orientation of larger ECM fibres is a concept not fully explored in the context of IPF. Our study exposes the possibility that this increased deposition of fibulin-1 and the increased secretion of periostin may

be altering the physical properties of fibroblasts and by extension, the lung tissue. Our study does not distinguish between extracellular and intracellular stores of fibulin-1, and therefore, further investigation around matrices from which cells have been removed is needed.

ECM proteins determine the biomechanical properties of the lung (Faffe et al. 2006). In this Chapter, treatment with TGFβ1 altered both the gross morphology and surface stiffness of Non-IPF and IPF fibroblasts. The median stiffness measurements of the fibroblasts were higher than those reported in the literature for lung fibroblasts (Liu et al. 2010) but this is likely due to the effect of fixation which has been shown to increase overall cell stiffness (Codan et al. 2013). Because live cells could not be investigated in our experimental set up, all cultures were fixed for the same length of time.

From the images of the fibroblast monolayer cultures morphological changes are visible following TGFβ1 stimulation in both Non-IPF and IPF fibroblasts. At this same timepoint (72 hours), there was an increase in fibulin-1 deposition only in the IPF fibroblasts. Furthermore, experiments with representative samples of Non-IPF and IPF fibroblasts indicate that treatment with TGFβ1 may increase the proportion of the cell's surface that demonstrates a high level of stiffness in both groups. However, the increase in stiffness was far greater in IPF fibroblasts compared to Non-IPF fibroblasts.

It is possible that the difference in the change of stiffness pattern is due to the increased deposition of fibulin-1. Further investigation, beyond the scope of this thesis is required to map the distribution of the fibulin-1 onto the stiffness maps of the fibroblasts to elucidate the potential relationship between the fibroblast stiffness and

fibulin-1 deposition. In addition, expanded experiments exploring the effect of TGFβ1 on cell stiffness are needed. Increased cell stiffness may also be a result of increased periostin deposition (Sidhu et al. 2010), or altered cytoskeletal arrangements (Jester et al. 1999) but these aspects were not studied as part of this thesis.

Experiments using fibulin-1 and/or periostin gene-silenced fibroblasts and stimulation with TGFβ1 could elucidate if these ECM proteins are playing a significant role in orchestrating the cell stiffness.

TGFβ1 is increased in the tissue of patients with IPF (Lepparanta et al. 2012) and would therefore be continuously present in the context of the disease. Our experiments indicate that fibroblast-secreted fibulin-1 may be driven into the ECM by TGFβ1. In the context of IPF, where fibroblast-secreted fibulin-1 is increased, this may result in increased stiffness of parenchymal fibroblasts and consequently, a stiffer lung which then results in decreased lung function.

**Table 4.5 Summary of results in Chapter 4**

| What is the effect of TGFβ1 on ECM proteins in primary parenchymal fibroblasts? | | Non-IPF | COPD | IPF |
|---|---|---|---|---|
| mRNA | Fibulin-1 | ↓ | ↓ | ↓ |
| | Periostin | ns | ns | ↑ |
| | Tenascin-C | ns | ns | ns |
| | Fibronectin | ↑ | ↑ | ↑ |
| Cell-secreted | Fibulin-1 | ↓ | ↓ | ↓ |
| | Periostin | ns | ns | ↑ |
| | Tenascin-C | ns | ns | ns |
| Cell-associated | Fibulin-1 | ns | ns | ↑ |
| Cell stiffness | Stiffness | ↑ | | ↑ |

Grey box indicates experiment not undertaken as part of this thesis
Primary parenchymal fibroblasts from patients with IPF (n=8) and age and gender matched patients without IPF (n=7) patients with COPD (n=4) were grown for 72 hours in 5% FBS/DMEM/1% pen-strep, quiesced in 0.1% FBS/DMEM/1% pen-strep for 24 hours and either left unstimulated (in fresh 0.1% FBS/DMEM/1% pen-strep) or stimulated for a further 72 hours with 10ng/mL TGFβ1 in 0.1% FBS/DMEM/1% pen-strep. Significant results are shown with the direction of the relationship between unstimulated and TGFβ1 treated cells indicated by the arrows (paired t-test, $p<0.05$).

Non-IPF fibroblasts were derived from the macroscopically normal tissue isolated from the tumour-free margin of resections from patients with non-small cell carcinoma.

COPD chronic obstructive pulmonary disease, IPF idiopathic pulmonary fibrosis, ns not significant, TGFβ1 transforming growth factor-beta 1, FBS foetal bovine serum, DMEM Dulbecco's Modified Eagle Medium, Pen-Strep penicillin-streptomycin

# Chapter 5. **General Discussion**

## 5.1 Summary and conclusions

Although pulmonary fibrosis is a feature of many interstitial lung diseases (ILDs), a diagnosis of idiopathic pulmonary fibrosis (IPF) carries with it the poorest prognosis. For many patients, disease progression is a foregone conclusion as there is no effective treatment for this disease and there have been no improvements in drug-based therapy in decades. At this moment in time, patients with IPF face a median survival time of 2-5 years but often without the knowledge of whether lung function decline will proceed rapidly or remain stable for years (Mura et al. 2012).

The fibrotic extracellular matrix (ECM) is now recognized as an important initiator, and driver, of the fibrotic process, but the contribution of individual components of the ECM has not been fully investigated. The experiments described in this thesis have, for the first time, identified the ECM protein fibulin-1 as a biomarker of disease progression in patients with IPF. Importantly, serum fibulin-1 levels were able to accurately identify patients with IPF who experienced rapid lung function decline independent of other predictors of survival (eg. baseline lung function).

IPF is a rare and heterogeneous disease. The diversity in the location from which patients' samples were sourced, coupled with the multiple patient materials that have been studied in this thesis highlights the consistency of the findings that fibulin-1 may be an important factor in the fibrogenic process. This study involved patients from three continents and researchers from seven institutions. Through these extensive networks of collaborators, investigations on patterns of ECM expression in serum,

lung lysates, fixed tissue, as well as isolated fibroblasts of patients with and without lung diseases was performed. Studies comparing fibroblasts derived from patients with various lung diseases have shown that differences in protein expression are maintained *in-vitro* (Marinkovic et al. 2012; Habiel and Hogaboam 2014) and therefore may reflect important differences in disease pathogenesis *in-vivo*. Building an extracellular foundation in any organ requires that component proteins are secreted from resident or invading cells in a soluble form and are then cross-linked into a deposited network by a cell-driven process with the help of existing "helper" molecules (Frantz et al. 2010). Some of these helper ECM proteins can be detected in the peripheral blood and serve as biomarkers of active fibrogenesis (Inoue et al. 2013).

Experiments in this study showed that fibulin-1 was a better biomarker of disease progression in IPF than the ECM proteins fibronectin, periostin and tenascin-C. This is an important finding considering that there are over 300 ECM proteins that could be dysregulated in the context of fibrosis (Booth et al. 2012) but not all are predictive of the disease process. In our study we confirmed that periostin, being the only other ECM protein identified as a potential biomarker for IPF (Naik et al. 2012), was also increased in both the serum and tissue of patients with IPF, but periostin did not predict disease progression these patients with IPF.

Fibulin-1 is secreted by lung fibroblasts and it is possible that the increased fibulin-1 in the serum of patients with IPF is a reflection of increased fibulin-1 production by fibrogenic fibroblasts. The basal expression of fibulin-1 was increased in fibroblasts derived from patients with IPF (IPF fibroblasts) compared to fibroblasts derived from patients without IPF (Non-IPF fibroblasts). This may also explain the source of the

increased fibulin-1 found in the lung tissue of patients with IPF. However, fibroblasts are not the only cell type known to be dysregulated in fibrosis and therefore other cells may be contributing the increased serum fibulin-1 levels in patients with IPF.

Parenchymal lung mechanics can be altered by increased ECM deposition (Faffe and Zin 2009) but it is the expression of α-smooth muscle actin (α-SMA) and stress fibres that determines the physical properties of fibroblasts themselves (Thoelking et al. 2010). Both ECM deposition and actin/stress fibre formation in fibroblasts are driven by transforming growth factor –β1 (TGFβ1) (Willis and Borok 2007) and therefore the effect of TGFβ1 on fibroblast-derived fibulin-1 was examined in the final Chapter of this thesis.

In this study, the effect of TGFβ1 was highly varied, keeping with the pleiotropic nature of the cytokine. This study is also the first to demonstrate that TGFβ1-induced cell-associated fibulin-1 production was limited to IPF fibroblasts and not Non-IPF or COPD fibroblasts. TGFβ1 increased the stiffness of both Non-IPF and IPF fibroblasts, albeit much more dramatically in IPF fibroblasts. This increase in stiffness may be attributed to increased cellular fibulin-1 deposition in IPF fibroblasts compared to Non-IPF fibroblasts, but this remains to be investigated in future studies.

## 5.2 Fibulin-1 is a biomarker of disease progression in IPF

Biomarkers of disease progression in patients with pulmonary fibrosis are needed to aid in current clinical management and treatment. As shown in Chapter 2, fibulin-1 is an attractive candidate biomarker as its levels are elevated in the serum, whole lung lysates, fixed lung tissue and isolated parenchymal fibroblasts of patients with IPF compared to subjects without IPF. Furthermore, patients with IPF had higher serum

fibulin-1 levels compared to patients with sarcoidosis and in patients with "other" ILDs examined, of which 74% (26 out of 35) had connective-tissue disease related ILD. In addition, patients with IPF were the only ILD category in this study to have serum fibulin-1 levels greater than subjects without lung disease, after adjustments for age, gender and smoking history were made.

While it would be reasonable to conclude that elevated fibulin-1 levels were a feature of IPF alone, a wide range of serum fibulin-1 levels is shared between ILDs. It is possible that serum fibulin-1 levels are simply a reflection of the fibrotic environment in a particular ILD patient and relate to a general state of active lung fibrosis, not a specific feature of IPF. In support of this hypothesis, this study also showed that serum fibulin-1 levels were inversely correlated with disease severity (as measured by the extent of fibrosis represented by the composite physiologic index) in patients with ILDs, including IPF. The follow up period of the patients in this study was a minimum of one year from blood sampling. Patients with IPF are the most likely to experience lung function decline or death (Demedts et al. 2001), and therefore a longer period of follow up for patients with other ILDs may be necessary in order to investigate the utility of serum fibulin-1 as a marker of disease progression in other ILDs, something which was outside the scope of this thesis.

The contribution of individual components of the ECM to lung mechanics is an area that is not well studied and this study was the first to recognize the correlation between increased levels of tissue fibulin-1 and decreased lung function in patients with IPF. Measuring the level of lung tissue fibulin-1 in two forms (whole lung lysates and fixed tissue sections) was important in determining the robustness of the results. The amount of elastic fibres, measured as the proportion of lung tissue that is

elastic fibre, predicts prognosis in IPF (Enomoto et al. 2013). Because fibulin-1 binds elastic fibres (Roark et al. 1995) it is possible that the increased levels of fibulin-1 are a manifestation of increased elastic fibre content.

A correlation between increased elastic fibre content and decreased FVC% has recently been reported in patients with IPF (Enomoto et al. 2013). Increased elastic fibre content was also predictive of disease progression in IPF (Enomoto et al. 2013). However, the correlation between tissue fibulin-1 and FVC% ($r = 0.9$, $p<0.05$) was much stronger than the correlation reported between elastic fibre content and FVC% ($r=0.5$, $p<0.01$) leading to the hypothesis that fibulin-1 is not merely a "passenger along for the ride" but may be directly influencing lung mechanics. The level of fibulin-1 may also directly affect the compliance, or elasticity, of the lung, although further experiments are needed to confirm this hypothesis.

The measured level of fibulin-1 in this study did not take into account any fragmentation that may have occurred post-translationally. Fibulin-1 in three types of patient materials was measured by western blot and therefore comparisons of the size of fibulin-1 between serum, whole lung lysates, and fibroblast-derived protein were possible. Western blots of serum showed four bands (weighing 100kDa, 73kDa, 50kDa and approximately 28-30kDa) for fibulin-1 but blots of lung lysates, fibroblast cell-secreted and cell-associated fibulin-1 showed only one band of 100kDa size.

All measurements of fibulin-1 in this study were based on the 100kDa size band. It is possible that the increase of fibulin-1 in the 100kDa band of the serum of patients with IPF is the contribution of fibulin-1 from the lung, in particular from the resident fibroblast. Previous studies of fibulin-1 in the literature have been measured by sandwich ELISA (Cangemi et al. 2011) and as a result, to our knowledge, this is the

first study to identify additional fragments of fibulin-1 in the serum that potentially could have bioactive properties of their own. Sequence analysis of the other size bands of fibulin-1 found in the serum are needed to identify these proteins in order to establish if they are fragments of fibulin-1, or artefacts of serum-derived proteins binding non-specifically to the fibulin-1 antibody used in the current studies. Should future studies determine that the bands of different sizes are fragments of fibulin-1, it would be interesting to determine if they also have roles in disease pathogenesis.

Fibulin-1 can be subjected to degradation by cathepsin D, a protease tasked with regulating apoptosis, which generates a bioactive fragment of approximately 65kDa named Neostatin that exhibits tumour suppressor activity (Xie et al. 2008). Enhanced cathepsin D protein has been reported in the alveolar epithelium of fibrotic lungs, particularly in regions of active proliferation (Kasper et al. 1996) and an inverse relationship between fibulin-1 and cathepsin D is seen in breast cancer (Pupa et al. 2004). Both fibrosis and cancer are instances of active ECM remodelling and therefore it would be interesting to investigate the consequences of fibulin-1 activity following degradation by other proteases involved in the lung fibrotic process such as matrix metalloproteinases (MMPs)(Song et al. 2013).

Dysregulation of fibroblast-derived ECM proteins in IPF is an area ripe for further study. It is incredible to realise that in a lifetime, the cells that populate an individual's organs are regenerated many times with little apparent loss of fidelity during normal homeostasis (Pellettieri and Sanchez Alvarado 2007). However, in the context of disease, studies of decellularized matrices indicate that there may be dozens of ECM proteins that are altered in the fibrotic lung (Booth et al. 2012) and in IPF, one of these dysregulated ECM proteins is periostin (Naik et al. 2012).

Therefore, it was important to investigate, in this study, whether other ECM proteins were also dysregulated in the context of IPF and could also be used as biomarkers of disease progression in addition to fibulin-1.

## 5.3 Dysregulation of ECM proteins in IPF

It is now recognized that the ECM plays an active role in determining cell behaviour such as morphology (Royce et al. 2009), proliferation (Krimmer et al. 2012), migration (Perumpanani et al. 1998), and signalling (Chiquet et al. 2003). The ECM has been shown to drive the fibrotic process by producing a fibrogenic positive-feedback loop by altering particular components of the ECM (Parker et al. 2014). It is critical to identify which of the nearly 300 proteins that constitute the ECM are the key players to target for future therapies. These proteins (fibulin-1, periostin, tenascin-C, fibronectin) influence one another (Williams and Schwarzbauer 2009; Kii et al. 2010) and in Chapter 3 it was demonstrated how relationships between these proteins may be important in understanding the pro-fibrotic environment in patients with fibrotic interstitial lung disease.

The results presented in this thesis support a model in which patients with fibrotic lung diseases can be stratified by their level of "fibrotic potential" and in addition to fibulin-1, the ECM proteins periostin and tenascin-C were also dysregulated in patients with IPF. For the purposes of this discussion, the fibrotic potential as been defined as the probability that a patient will experience a progressive decline in lung function over the course of their disease.

Within 5 years of diagnosis, 44% of all patients with IPF are expected to die, compared with only 2% of patients with sarcoidosis (Demedts et al. 2001) and

therefore, in keeping with our definition, patients with IPF have the highest fibrotic potential whereas those with sarcoidosis have the lowest. The fibrotic potential of a patient may be quantified by sampling serum levels of any number of ECM proteins in patients with ILD.

In this study, 45% (75/167) of patients with ILD exhibited increased levels of at least one of the four serum ECM proteins studied. Pearson's chi-squared test was used to assess whether there was a relationship between the number of patients who had a high level of at least 1 ECM protein and the particular ILD group. The proportion of patients who had high levels of serum ECM proteins differed between disease groups (Table 5.1). A high level of serum ECM protein was defined as greater than or equal to the mean + 2 standard deviations level of that protein measured in the healthy controls (n=17). If patients with IPF had the highest fibrotic potential, then it would make sense that more than half (52%) of the patients with IPF exhibited high levels of at least one of the four ECM proteins we studied (Table 5.1).

Furthermore, patients with IPF that had a high level of at least one of the ECM proteins studied were also more likely to progress within one year of blood draw (Table 5.2).

**Table 5.1 The number of patients with a high serum level of at least one ECM protein is greatest in IPF**

| Disease Category | (n) | Fibulin-1 | | Periostin | | Tenascin-C | | Fibronectin | | **At least 1 ECM*** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Count | % | Count | % | Count | % | Count | % | **Count** | **%** |
| **Non-diseased Control** | 17 | 1 | 6% | 1 | 6% | 0 | 0% | 1 | 6% | **2** | **12%** |
| **Sarcoidosis** | 12 | 0 | 0% | 2 | 17% | 6 | 50% | 2 | 17% | **4** | **33%** |
| **HP** | 32 | 9 | 28% | 6 | 19% | 18 | 56% | 5 | 16% | **16** | **50%** |
| **"Other" ILD** | 35 | 10 | 29% | 6 | 17% | 11 | 31% | 4 | 11% | **16** | **46%** |
| **IPF** | 71 | 28 | 39% | 15 | 21% | 22 | 31% | 14 | 20% | **37** | **52%** |
| **Total** | 167 | 48 | | 30 | | 57 | | 26 | | 75 | 45% |

The means and standard deviations (SD) of serum levels of the ECM proteins were calculated from n=17 non-diseased controls with no history of lung disease. A high level of ECM protein was defined as greater than or equal to the mean+ 2SD of that protein.
Those patients who had a high level of serum ECM protein were counted individually.
% is defined as the number of patients who had a high level of ECM protein expressed as a percentage of the total number of patients within each disease category.
***At least 1 ECM** is defined as the number of patients who had a high level of at least one of the four ECM proteins.
**Pearson Chi-Square value 9.771, degrees of freedom 4, p=0.044**
”Other” refers to patients with connective tissue-disease related ILD (n=26), Non-specific interstitial pneumonia (n=4), lymphangioleiomyomatosis (n=4), and drug-induced ILD (n=1).
ECM extracellular matrix, ILD intersitital lung disease, IPF idiopathic pulmonary fibrosis, HP hypersensitivity pneumonitis

**Table 5.2 Patients with IPF who had a high ECM level were more likely to progress within 1 year of blood draw.**

| | | Normal ECM levels | High ECM levels | Total patients | Pearson Chi-Square p-value |
|---|---|---|---|---|---|
| Non-IPF ILD | Stable | 9 | 11 | 20 | 0.214 |
| | Progressed | 4 | 12 | 16 | |
| Total Non-IPF | | 13 | 23 | 36 | |
| IPF | Stable | 15 | 6 | 21 | 0.025 |
| | Progressed | 11 | 16 | 27 | |
| Total IPF | | 26 | 22 | 48 | |

The means and standard deviations (SD) of serum levels of fibulin-1, periostin, tenascin-C and fibronectin was calculated from n=17 non-diseased controls with no history of lung disease.
A high level of an ECM protein was defined as greater than or equal to the mean + 2SD level of that protein. A patient was considered to have high ECM levels if any one of the four ECM protein levels was high.
Patients that did not have high serum levels of any of these ECM proteins were considered to have normal ECM levels.
Progression was defined as having a significant fall in lung function (defined as ≥10% relative fall in FVC%, ≥15% relative fall in $DL_{CO}$%, or death one year of blood draw).
ILD interstitial lung disease, IPF idiopathic pulmonary fibrosis, ECM extracellular matrix

Serum levels of periostin and tenascin-C also inversely correlated with lung function parameters in patients with ILDs. Like a concentration gradient, it may be that increased systemic levels of ECM proteins act to tilt the patient's circulatory microenvironment towards a state of enhanced ECM deposition, outside of *de novo* ECM production. Fibulin-1 incorporation into the ECM requires fibronectin, and increasing amounts of fibronectin in the matrix also increases the amount of fibulin-1 deposited (Godyna et al. 1995). Increases in systemic levels of particular ECM proteins may drive the increased deposition of that ECM protein, and potentially also proteins that associate with the protein of increased levels, into the lung as more "fibrotic building blocks" are available for use during wound healing events.

One of the mechanisms driving the progression of IPF is thought to be propagated through constant damage to the lung (Wilson and Wynn 2009), in which vascular homeostasis plays a critical role. The initial stage of wound healing is haemostasis and in concert with fibulin-1 (Godyna et al. 1996), it is fibronectin and fibrin that form the initial thrombus, or "plug", that seals the wound (Stenman et al. 1980). Fibronectin fibre assembly is of particular importance because collagen fibres do not form without the presence of fibronectin (Sottile and Hocking 2002). This provisional matrix provides the seed structure for the deposition of collagen that follows (Hernnas et al. 1992). In this manner, it is possible that the increased amounts of fibulin-1, produced by IPF fibroblasts, could be contributing to fibrosis via the role of fibulin-1 in the initial clot formation that underlies all fibrosis (Tran et al. 1995). In this study increased levels of fibronectin in the patients with ILDs were not seen. It is possible that whilst fibulin-1 and fibronectin are known to bind to each other in tissue (Godyna

et al. 1995), this does not necessarily mean that the two molecules bind in the circulation.

In the later stages of wound healing, after the initial deposition of the individual subunits of collagen, cross-linking of the collagen strands occurs through the maturation process of the fibres. During this process, myofibroblasts replace the fibronectin in the provisional matrix with decorin. Decorin is a "C" shaped molecular bridge that is placed between collagen fibrils as a "spacer," ensuring uniform distance between collagen fibres (Weber et al. 1996). Fibroblasts from patients with pulmonary fibrosis also produce more decorin than fibroblasts from non-fibrotic subjects (Westergren-Thorsson et al. 2004). This study did not investigate the role of decorin in the tissue of patients with IPF and future work is necessary to elucidate the relationship between decorin and the ECM proteins studied here. However, similarly to decorin, periostin functions as a molecular spacer in collagen fibrillogenesis (Norris et al. 2007) and can also alter fibronectin fibres by acting as a bridge linking multiple fibronectin fibres (Kudo 2011).

An essential part of ECM remodelling is collagen fibril formation. Collagen fibril formation requires the correct intracellular formation of ECM component proteins in the endoplasmic reticulum in a process that involves many molecular chaperones for proper folding and secretion (Lamande and Bateman 1999). Once in the extracellular space, the mechanisms involved in tissue-scale ECM formation are complex and often require the assistance of fibril-associated molecules that are targeted to the surface of collagen precursors (Canty and Kadler 2005). The mechanisms driving collagen fibril formation are outside the scope of this thesis however this study highlighted a novel link between fibulin-1 and periostin in lung tissue from patients with IPF. Further

investigation into the role of fibulin-1 in collagen fibril formation, via the shared correlation between fibulin-1 and periostin and periostin and collagen in lung tissue from patients with IPF are warranted.

Whilst levels of tissue periostin and tissue total collagen were also increased in lung tissue from patients with IPF, levels did not correlate with lung function in these patients with IPF. Interestingly, tissue periostin did correlate strongly with tissue total collagen, as would be expected from prior reports in the literature (Takayama et al. 2006). Although collagen is a major determinant of lung function (Suki et al. 2005), it is possible that the increases seen in periostin/total collagen deposition in the tissue of patients with IPF was not as influential on lung function as the increases seen in fibulin-1 deposition. Fibulin-1 may have a strong role in determining lung function because of its role in elastic fibre formation (Sasaki et al. 1999). Elastic fibres are another major determinant of lung function (Faffe and Zin 2009).

A greater mechanistic understanding of the processes regulating and driving site-specific fibrogenesis would allow for the development of anti-fibrotic treatments potentially capable of "resetting" the dysregulated ECM and restoring normal lung architecture and function. Future studies investigating the precise manner in which the network of proteins that makes up the ECM are aligned relative to each other are needed.

## 5.4 Fibulin-1 production in primary parenchymal fibroblasts is increased by TGFβ1 in patients with IPF

Fibroblasts from patients with IPF inherently produce increased levels of fibulin-1, at both the gene and protein levels. Chapter 3 was concluded by showing that none of

the other three proteins studied in this thesis (periostin, tenascin-C, or fibronectin) shared this same characteristic. Fibroblasts are capable of altering specific ECM proteins in response to mechanical stress even in the presence of TGFβ1 (Chiquet et al. 2003), alluding to parallel production of ECM proteins, as well as growth factors, in response to mechanical stress.

TGFβ1 is a potent inducer of ECM proteins (Zanotti et al. 2010). TGFβ1 is also released from activated platelets in the first stages of wound healing (Grainger et al. 1995). The earlier hypothesis that increased circulating fibulin-1 was driven into the matrix during wound healing events was extended in Chapter 4 by including a study of TGFβ1 as an inducer of fibulin-1 deposition. The data presented here lead to the hypothesis that in a healthy individual, after an injurious event to the lung, in which blood vessels are damaged, TGFβ1, perhaps released from platelets, drives fibulin-1 from the blood into the wound. In a patient with IPF, increased levels of fibulin-1 may be deposited in the tissue because of (1) increased levels of circulating fibulin-1, and/or (2) increased production of fibulin-1 by fibrotic fibroblasts.

TGFβ1 increased fibronectin mRNA in lung fibroblasts this study (Chapter 3) and has been shown to increase fibronectin protein deposition by lung fibroblasts (Patel et al. 2012). It is possible that TGFβ1-induced fibronectin deposition subsequently increases fibulin-1 deposition. Fibronectin regulates latent TGFβ1 by controlling the assembly of the latent-TGFβ1-binding protein-1 into the matrix (Dallas et al. 2005). There is extensive evidence that fibronectin is important in the context of fibrotic disease (White and Muro 2011) but its significance may actually be due in part to how it interacts with other proteins (Muro et al. 2008), for example fibulin-1.

Upon activation from its latent state, TGFβ1 stimulation increases other pro-fibrotic cytokines such as vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) (Lee 2012). Levels of CTGF in the serum have also been reported as a biomarker of disease progression in IPF (Kono et al. 2011) and another extension of this work could be to investigate the effect of CTGF on fibulin-1. Other cytokines such as the tumour cytokine CXCL1/GROα have been reported to decrease fibulin-1, resulting in increased cell migration of prostate cancer cells (Kuo et al. 2012). Additional cytokines may prove to also regulate fibulin-1 deposition, the study of which could also broaden the results of this thesis.

Gene levels of pro-inflammatory cytokines like interleukin (IL)-6 are increased in patients with IPF (Pantelidis et al. 2001) and predictive of disease progression (De Lauretis et al. 2013). Furthermore, although obvious inflammation is absent in patients with established IPF (Keane 2008), TGFβ1 has been shown to induce IL-6 mRNA in primary human lung fibroblasts (Eickelberg et al. 1999), and in this study, also induced IL-6 protein. IL-6 contributes to proliferation and reduces apoptosis in a subpopulation of fibroblasts in patients with IPF and is therefore important in disease progression (Habiel and Hogaboam 2014).

Most relevant to our study is that IL-6 can regulate ECM deposition in the context of an already disordered ECM. Mice with fibrillin-1 deficiency, a model of Marfan's syndrome which is a connective tissue disorder resulting in heart defects, are protected from ECM degeneration when IL-6 is also knocked out (Ju et al. 2014). Fibrillin-1 binds fibulin-1 (El-Hallous et al. 2007), and therefore, it is possible for IL-6 to indirectly affect fibulin-1 deposition into the ECM and could be explored as an extension of this study.

The deposition of particular ECM proteins gives each tissue ECM a unique topography that is generated by an exceedingly complex interaction between epithelial, endothelial, adipocyte and fibroblast cells (Frantz et al. 2010). This study concluded by examining morphological, and physical, changes in the topography of lung fibroblast cells after TGFβ1 stimulation, from which the importance of differences in ECM deposition was inferred.

It was hypothesized that increased fibulin-1 deposition into the ECM decreased lung function due to the role that fibulin-1 plays in determining the orientation of larger structural fibres such as fibrillin-1, fibronectin (Cooley et al. 2008), and elastic fibres (Roark et al. 1995). This was supported by the results presented in Chapter 2, which indicated a strong correlation between the level of fibulin-1 deposited in lung tissue and the lung function of that patient with IPF. In addition it was concluded, from the data presented in Chapter 3, that the relationship between amount of ECM protein and lung function was not a feature of just any ECM protein, as periostin did not possess this characteristic despite being increased in the same tissue samples from patients with IPF. Differences in lung tissue levels of fibulin-1 between patients with and without IPF were mirrored in the fibroblasts derived from the aforementioned patients.

Fibroblasts from a patient with IPF were stiffer than fibroblasts from a patient without IPF. Furthermore, while TGFβ1 stimulation increased the stiffness of both Non-IPF and IPF fibroblasts, TGFβ1 stimulation increased fibulin-1 production in IPF fibroblasts but not in Non-IPF fibroblasts. The mechanism underlying fibulin-1 deposition by fibroblasts may be regulated by TGFβ1. In support of this possibility, greater levels of TGFβ, co-localised with ECM proteins that bind fibulin-1, namely

fibronectin and fibrillin-1, are found in the lungs of patients with IPF (Lepparanta et al. 2012).

In a healthy lung, an injury starts a chain of events that terminates in the restoration of the normal lung architecture and a return to normal lung function. Any number of control-points that lie along this chain of events may become dysregulated in the context of fibrotic ILDs that progress to lung failure. IPF is thought to be driven by activated fibroblasts that deposit excessive amounts of ECM in an aberrant wound response. Understanding this mechanism is of importance because IPF carries the greatest public burden due to its lack of treatment and high rate of mortality (Raghu et al. 2011).

A summary of the results in this thesis is found in Table 5.3. The novel findings presented in this thesis are illustrated in Figure 5.1. Increased deposition of fibulin-1 into the tissue ECM may result in decreased elasticity as fibulin-1 is a component of elastic fibres. Increased deposition of fibulin-1 may result in the overall stiffening of the lung, and eventual loss of function. Levels of circulating fibulin-1 predicted disease progression in patients with IPF and could be a useful clinical biomarker. Insights into the precise role of fibulin-1 in the ECM and its effect on fibroblasts could lead to new therapies for drug development and targeting.

**Table 5.3 Summary of thesis**

| | Research Question | | | | |
|---|---|---|---|---|---|
| | Biomarker Study | **Fibulin-1** | **Periostin** | **Fibronectin** | **Tenascin-C** |
| 1 | Is the biomarker increased in the serum of patients with IPF compared to controls? | Yes* | Yes | No | No |
| 2 | Does the biomarker predict progression in patients with IPF? | Yes* | No | No | No |
| 3 | Is the biomarker increased in the tissue of patients with IPF compared to controls? | Yes* | Yes | No | |
| 4 | Do fibroblasts from patients with IPF produce increased mRNA of the biomarker? | Yes* | No | No | No |
| 5 | In patients with IPF, do fibroblasts produce increased levels of the soluble biomarker protein? | Yes* | No | | No |
| 6 | In patients with IPF, do fibroblasts produce increased levels of the cellular biomarker protein? | Yes* | | | |
| 7 | Does TGFβ1 increase the biomarker mRNA in fibroblasts from patients with IPF? | No* | Yes* | Yes | No |
| 8 | Does TGFβ1 increase the levels of deposited biomarker protein in fibroblasts from patients with IPF? | Yes* | | | |
| | Matrix Stiffness Study | **Non-IPF** | | **IPF** | |
| 9 | Are fibroblasts from patients with IPF stiffer? | n/a | | Yes* | |
| 10 | Does TGFβ1 increase the stiffness of fibroblasts? | Yes | | Yes* | |

*indicates novel findings

Grey boxes indicate research questions not undertaken as part of this thesis.

mRNA messenger ribonucleic acid, IPF idiopathic pulmonary fibrosis

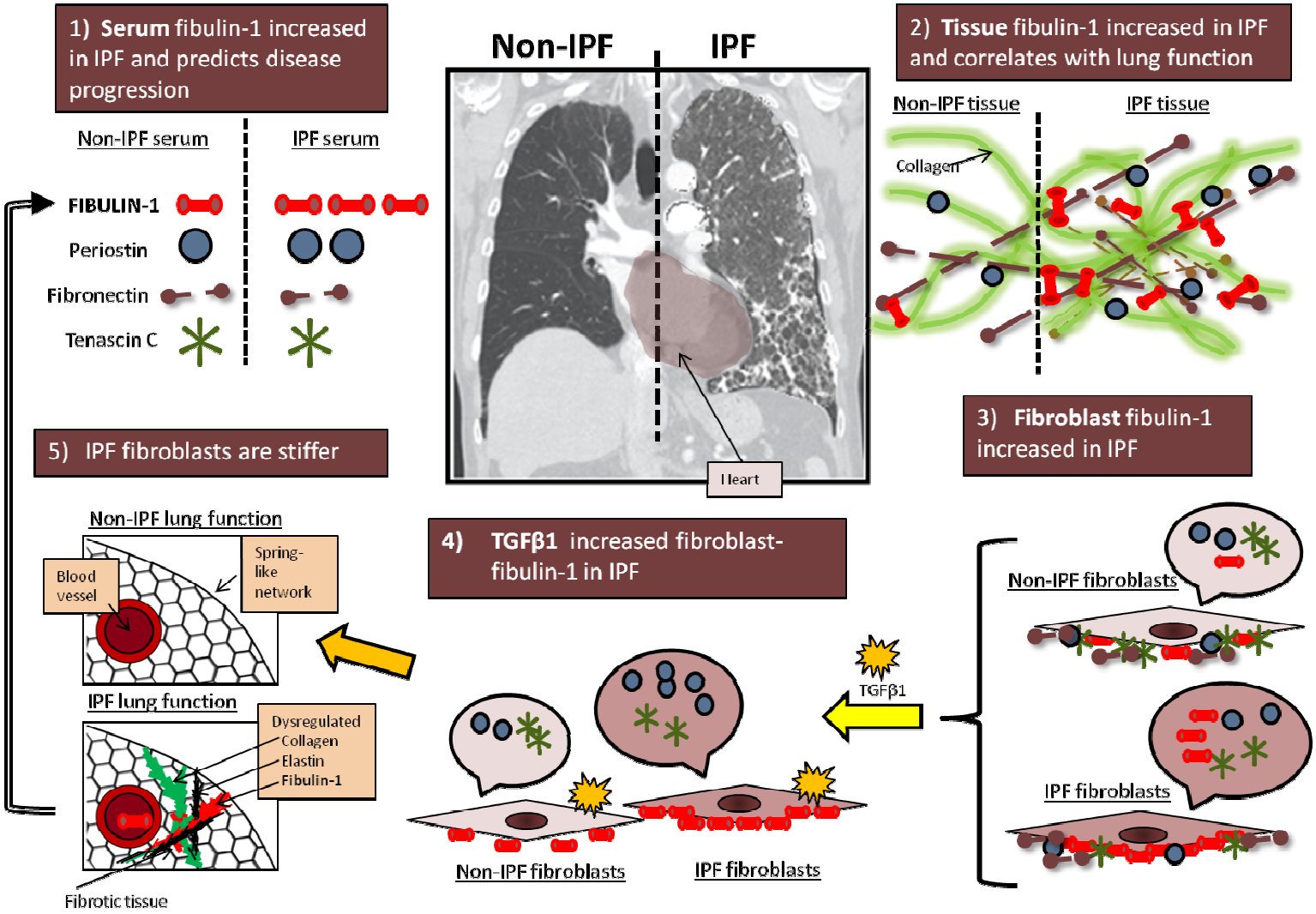



**Figure 5.1 Summary of the results presented in this thesis.**

Five novel findings presented in this thesis are illustrated.

High-resolution computed tomography scans from a patient without (http://www.ers-education.org/media/83651/ers_handbook_2nd_samplesection.pdf, accessed 02-06-14) and with IPF (reproduced with permission from (Raghu et al. 2011)) were used to compare the differences in lung composition. Lighter areas are more solid.

Double-lined black arrow represents a possible source of increased fibulin-1 in the serum of patients with IPF.

## 5.5 Future Directions

The main limitation in this study was the inability to study serial samples of serum in patients with ILDs. Investigation of the stability of fibulin-1 levels would be necessary to confirm the findings presented in this thesis. Furthermore, the results described herein would be strengthened following validation in a second study of an alternate population of patients with IPF.

Properties of ECM proteins differ between their soluble form and their cell-associated form and ECM proteins can alter collagen fibrogenesis (Flynn et al. 2010). Fibroblasts from subjects without IPF showed a different pattern of stiffness compared to fibroblasts from patients with IPF (Figure 5.1). Fibroblasts from subjects without IPF had a majority of points of stiffness on the normal end of the stiffness scale whereas fibroblasts from patients with IPF had a shift in the median towards fibrotic levels of stiffness. An additional limitation in the experimental set up required that the cells investigated using AFM in this thesis needed to be fixed prior to analysis.

Further follow up work would be required to strengthen these observations. Fibroblasts derived from fibrotic disease states are known to respond to matrices of differing stiffness (Marinkovic et al. 2012), but no study has investigated the inherent differences between IPF fibroblasts and normal fibroblasts.

The differences described above may relate to the differential expression of ECM proteins, in particular fibulin-1. It would be interesting to see if knockdown of fibulin-1 has an effect on the stiffness of the overall lung matrix. One way to study this would be the use of AFM on lung tissue from fibulin-1 heterozygous knockout mice. A

heterozygous knockout would be necessary as homozygous fibulin-1 knockouts are embryonically lethal (Kostka et al. 2001). Mouse models with and without fibulin-1 would also allow for the study of the effect of fibulin-1 on lung mechanics *in vivo*.

Another way to study the effect of fibulin-1 in cell stiffness would be through the use of a transfected cell line such as the one that was used as a positive control in this study. The HT1080 fibrosarcoma line does not produce fibulin-1 and was transfected with the C-isoform of fibulin-1 to study the anti-angiogenic function of fibulin-1 (Xie et al. 2008). Differences in the stiffness between HT1080 cell lines transfected with and without the fibulin-1 gene would allow for a direct comparison of the impact of fibulin-1 on cell stiffness both in the presence and absence of cytokines like TGFβ1. TGFβ1 is not the only cytokine that induces fibulin-1. In the cancer literature, oestrogen (Nakamoto et al. 2005) and progesterone (Moll et al. 2002) both can induce fibulin-1 in ovarian cancer and endometrial stromal cells respectively. Furthermore, expression of fibulin-1 is increased in preeclampsia, a pregnancy-specific syndrome of which proteinuria (excess serum proteins in the urine) is characteristic (Liu et al. 2011). Therefore, it would be worthwhile investigating if other cytokines can induce fibulin-1 in the context of fibrotic lung disease.

Dysregulation of fibulin-1 may have subsequent effects on the deposition of other ECM proteins as the creation of this complex network involves many players. The relationship between ECM proteins has not been extensively studied and as shown in the preceding Chapters, ECM proteins correlate with each other. Future work could extend the aforementioned experiments of cell stiffness to include studies on the deposition patterns of other ECM proteins when fibulin-1 is knocked out. Such experiments would go towards elucidating if fibulin-1 is a driver of the fibrogenic

process or is merely a bystander, increased through association with some of the key players in the progression of fibrosis. Ultimately, a greater understanding of fibulin-1 and the mechanisms that drive fibrosis would allow for more targeted treatments in pulmonary fibrosis.

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