# THE ROLE OF GALECTIN-3 IN THE DEVELOPMENT OF IDIOPATHIC PULMONARY FIBROSIS

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PhD Thesis

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# Abstract

**Introduction**

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease with a poor prognosis. Currently there is no cure for IPF but there are two licenced therapeutic drugs which can only slow down the progression of fibrosis. Transforming growth factor β (TGFβ) plays a crucial role in the pathogenesis of IPF. The β-galactoside binding lectin galectin-3 promotes fibrosis through modifying the activity of TGFβ. However, the precise mechanism of galectin-3 promoting fibrosis currently unknown. A galectin-3 inhibitor (TD139) is currently in clinical trials for the treatment of IPF. Additionally, galectin-3 is thought to play an important role in TGFβ signalling, where loss of galectin-3 in cell prevents TGFβ induced effects. The mechanism of this is also unclear.

**Methods**

Immunohistochemistry (IHC) was used to stain for galectin-3 in lung sections from patients with IPF and mice treated with bleomycin. Human lung fibroblasts (HLFs) isolated from healthy patients and IPF patients, and immortalised human bronchial epithelial cells (IHBECs) were used in the study. They were treated with galectin-3 inhibitor TD139 and then stimulated with recombinant human galectin-3 (rhGal-3). To delineate the possible mechanism of galectin-3 promoting TGFβ activity HFLs were stimulated with LPA and the PAR1 agonist SFLLRN and treated with αvβ5

antibody, β1 inhibitor or TD139. HLFs were treated with β1 inhibitor or αvβ5 antibody and stimulated with rhGal-3. Solid-phase binding assays were used to investigate whether galectin-3 binds to components of the latent TGFβ complex. Co-immunoprecipitation studies were undertaken to investigate galectin-3 and αv integrin subunit interaction. HLFs and IHBECs were treated with TD139 and stimulated with recombinant TGFβ. Protein expression of phosphorylated Smad2 (pSmad2) was used as a measurement of TGFβ signalling and was assessed by western blot. qPCR was used to measure galectin-3 at the mRNA level in HLFs treated with TGFβ.

**Results**

IHC showed an increase in galectin-3 expression in patients with IPF and in the mice treated with bleomycin; where expression was increased in airway epithelial cells, fibroblasts, and macrophages. RhGal-3 stimulation increased pSmad2 levels in HLFs but not in IHBECs. This was inhibited by TD139 treatment. B1 and αvβ5 antibody blocked LPA induced pSmad2 increases. Galectin-3 inhibition with TD139 blocked both LPA and SFLLRN induced smad2 phosphorylation. B1 inhibition and αvβ5 antibody treatments had no effect on rhGal-3 induced pSmad2 increases. Solid-phase binding assay showed that galectin-3 binds to LTBP1 of the TGFβ latent complex. Co-immunoprecipitation studies revealed that galectin-3 associates with αv integrin subunit. Treatment with TD139 inhibited recombinant TGFb induced smad2 phosphorylation in HLFs but not in IHBECs. TGFβ stimulation significantly increased galectin-3 expression at the mRNA level in IPF HLFs but not control HLFs.

**Conclusion**

Results show that galectin-3 promotes TGFβ signalling in fibroblasts but not in epithelial cells. It is thought there are 3 possible mechanisms by which galectin-3 promotes TGFβ activity. (1) galectin-3 forms a lattice complex with integrins to enhance integrin-LAP binding. (2) galectin-3 binding to the LTBP1 region of the large latent TGFβ complex and tightly anchoring the latent complex to the ECM resulting in little traction force needed for TGFβ to be released. (3) galectin-3 forming lattices between αv integrins and the latent TGFβ complex to increase accessibility of the integrin to bind to LAP leading to TGFβ release. Thus, the galectin-3-integrin site could be used as a therapeutic target for IPF treatment.

# Acknowledgments

I am extremely grateful to The Nottingham Pathology Node and Galecto Biotech for funding my PhD project.

I would like to thank everyone within the Respiratory Medicine department for their constant help and support during my 3 years.

I cannot thank my supervisor enough, Professor Gisli Jenkins. Thank you for your endless help, advice and mentorship throughout my PhD and after. Your enthusiasm for research, motivation and knowledge has truly inspired me to continue doing research.

I want to thank my Grandparents, my Mum and Dad, and Akshay for their endless love and encouragement. They pushed me and instilled qualities in me such as to always work hard, pursue to always learn and develop, and perseverance. All without which I couldn't have completed my PhD.

Lastly, I want to thank Sunanda, the greatest partner I could have ask for. You helped me to get into my PhD, you helped me during my PhD, you helped me to get an amazing job after my PhD. Without you this dream of mine would have never come true.

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# Chapter 1: Introduction

# Investigate the role of Galectin-3 in the Development of Idiopathic Pulmonary Fibrosis

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, irreversible and progressive fibrotic disease of the lung. Due to the lack of effective therapies the prognosis for IPF is poor, where median survival from time of diagnosis is 2-3 years (Ley, Collard and King, 2011). It is a condition of unknown aetiology, however, a few risk factors such as cigarette smoke and viral infections have been shown to increase the risk (Baumgartner *et al.*, 2000). The disease is characterised clinically by progressive breathlessness upon exertion and a dry irritating cough. Histologically, IPF is characterised by features of usual interstitial pneumonia (UIP) which includes fibroblastic foci containing fibroblasts and myofibroblasts, and honeycombing (Katzenstein and Myers, 1998).

The pathogenesis of IPF is poorly understood with the disease being formerly attributed to chronic inflammation playing a central role (Keogh and Crystal, 1982). Anti-inflammatory strategies have shown to be ineffective treatments for IPF and inflammation is not a major feature in pathological tissue specimens (Johnson *et al.*, 1989; Raghu *et al.*, 1991; Mapel, Samet and Coultas, 1996; Katzenstein and Myers,

1998; Gross and Hunninghake, 2001; Selman, King and Pardo, 2001). Thus, the current hypothesis of the disease pathogenesis involves repeated injury to the alveolar epithelium in a genetically susceptible individual, followed by ineffective wound healing responses (Selman, King and Pardo, 2001). The causes of this recurrent injury are unknown but might include cigarette smoke, wood and metal dust (Hubbard *et al.*, 1996). Furthermore, the atypical wound healing seen in IPF in response to injury is caused by the dysregulation in the release and action of cytokines such as transforming growth factor β (TGFβ) and platelet derived growth factor (PDGF) (Khalil *et al.*, 1991; Bonner, 2004).

Currently there are only two drugs used to treat IPF: Pirfenidine and Nintedanib. Though the two drugs have been shown to slow down the progression of IPF, they fail to reverse the disease (Azuma *et al.*, 2010; Taniguchi *et al.*, 2010; Noble *et al.*, 2011; Richeldi *et al.*, 2011; King *et al.*, 2014) . The only cure for IPF is lung transplant. Thus, highlighting the need for further research into understanding the pathogenesis of the disease and the clinical need for a therapeutic drug.

# Idiopathic Pulmonary Fibrosis

## Clinical features of IPF

IPF is the most common form of idiopathic interstitial pneumonias (IIPs). IIPs are a group of diffuse parenchymal lung diseases which was first classified in 2000 and further revised in 2011 by the American Thoracic Society and European Respiratory Society (ATS/ERS) (Travis *et al.*, 2002; Raghu *et al.*, 2011). They are categorised according to their clinical and histological features which lead to different treatments and outcomes. IIPs are characterised by hypertrophy of the alveolar septa are thought to be can due to inflammation (Travis *et al.*, 2002; Raghu *et al.*, 2011, 2018). Furthermore, the progressive nature of the disease leads to remodelling of the lung tissue through fibrosis.

The ATS/ERS defined IPF as a chronic and progressive interstitial pneumonia with an unknown cause; which is characterised by histopathological patterns of UIP taken from lung biopsy tissue, and patterns of UIP seen on high-resolution computed tomography (HRCT) (Raghu *et al.*, 2011, 2018). The main histopathological feature of UIP is fibrotic scarring that is temporally and spatially heterogenous with areas of normal lung (Raghu *et al.*, 2018). Additionally, interstitial lymphoplasmacytic inflammation is also visualised. Areas of honeycombing, defined as cystically dilated airspaces lined by bronchiolar epithelium, are also a feature of UIP (Raghu *et al.*, 2018). These honeycomb areas in the fibrotic lung are often filled with mucin and inflammatory cells (Raghu *et al.*, 2018). Another feature in UIP is the presence of fibrotic foci (Raghu *et al.*, 2018). These are concentrated areas of active fibroblasts and myofibroblasts arranged in a linear fashion on a background of dense collagen

deposition. Although IPF is defined by the presence of the histological lesion of UIP this can only be obtained by lung biopsy and in many cases, it isn't safe to undergo biopsy, so diagnosis is made by High Resolution Computerised Tomography (HRCT) (Raghu *et al.*, 2018).

Due to the difficulties obtaining biopsies and the challenges discriminating microcystic honeycombing radiologically the integration of clinical, radiological and histological information the role of the multi-disciplinary team has developed considerably (Raghu *et al.*, 2018). Patterns of UIP on HRCT is characterised by reticular opacities with no or minimal ground glass opacity, which is often associated with traction bronchiectasis (Raghu *et al.*, 2018). Presence of honeycombing is also critical in diagnosis (Raghu *et al.*, 2018). Honeycombed areas (typically 3-10mm diameter) are sub-plural cystic airspaces with well-defined walls (Hansell *et al.*, 2008). Honeycombing can be classified into microcytic honeycombing, macrocytic honeycombing, mixed microcytic and macrocytic honeycombing, and combined emphysema and honeycombing (Hansell *et al.*, 2008; Raghu *et al.*, 2018). UIP usually exhibits mixed microcytic and macrocytic honeycombing, indicating that the disease is a result from repeated damage to the alveolar epithelium (Raghu *et al.*, 2018).

IPF is diagnosed through histopathological patterns of UIP from lung biopsies, radiological UIP patterns, and the exclusion of other known causes of interstitial lung diseases (ILD). Hence, the diagnosis of IPF requires strong interactions

between clinicians, pathologists, and radiologists- multi-disciplinary approach (Raghu *et al.*, 2011, 2018).

Due to the progressive nature of the disease, IPF is fatal with a very poor prognosis. The fibrosis leads to thickening of the interstitium of the alveolar wall, resulting in reduced lung compliance (Plantier *et al.*, 2018). This reduced distensability of the lungs mean that affected patients have a markedly reduced forced vital capacity (FVC), low forced expiratory volume in 1 second ($FEV_1$), and a reduced total lung capacity (TLC) (Plantier *et al.*, 2018).

The arterial $P_{O2}$ and $P_{CO2}$ in patients with IPF are typically reduced resulting in patients suffering from hypoxemia (Plantier *et al.*, 2018). The hypoxemia is attributed to the ventilation-perfusion ($V_A/Q$) mismatch (Plantier *et al.*, 2018). Hence, patients present with shortness of breath; with shortness of breath becoming more significant upon exercise (Martinez *et al.*, 2005; Swigris *et al.*, 2008).

The progressive and irreversible nature of the disease and the reduced gas exchange consequently mean that lung function of affected patients will deteriorate over the course of few years, eventually leading to respiratory failure and death. Thus, the mean survival from diagnosis is 2-3 years (Ley, Collard and King, 2011). However, it is difficult to predict how the condition will progress as the clinical course of affected patients is heterogeneous. Some patients show a slow and gradual lung

deterioration while some others show an abrupt and rapid deterioration, which can be from an unidentifiable cause known as acute exacerbations (AE) (Ambrosini *et al.*, 2003; Song *et al.*, 2011). Worse prognosis of the disease is often associated with old age, pulmonary hypertension, and smoking history.

Many patients often follow a slow and stable progressive course, whereas some patients can follow a rapid disease course. It is often seen, patients in the rapid disease course group are cigarette smokers (King, Pardo and Selman, 2011). Hence, patients with acute exacerbations have poorer outcomes (Collard *et al.*, 2007, 2016). An acute exacerbation is defined as a rapid deterioration of the disease of any identifiable cause (Collard *et al.*, 2007, 2016).

## Epidemiology and risk factors

The prevalence of IPF continue to rise and the incidence increase with age (Raghu *et al.*, 2006; Hutchinson *et al.*, 2015). The British Lung Foundation (BLF) estimated that in 2012 that 32,500 people in the United Kingdom had IPF (British Lung Foundation, 2019). IPF occurs typically in the elderly, where the median age at diagnosis is 66 years (Meltzer and Noble, 2008; King, Pardo and Selman, 2011; Hutchinson *et al.*, 2015). IPF predominantly affects males, where 16 males and 9 females for every 100,000 are newly diagnosed with IPF every year (British Lung Foundation, 2019). 5,292 people in 2012 died from IPF (90% of these patients were above 65 years of age), up from 3,964 in 2008 (British Lung Foundation, 2019).

The cause of IPF is still poorly understood, the current evidence suggests that IPF occurs from a combination of genetic and environmental risk factors. It is thought that the prominent risk factors are likely to be inhaled agents such as cigarette smoke and dusts from woods and metals (Taskar, 2006). Thus, the inhaled agents are thought to lead to the injury of the epithelium and epithelial apoptosis. These environmental factors together with a genetic predisposition causes an abnormal epithelial cell response triggering epithelial cell apoptosis and dysregulation in the repair response, ultimately resulting in fibrosis (Gross and Hunninghake, 2001; Selman, King and Pardo, 2001; Martinez *et al.*, 2017; Richeldi, Collard and Jones, 2017).

Gastroesophageal reflux has been linked to a number of respiratory diseases including IPF (Richeldi, Collard and Jones, 2017). The repeated chronic injury from gastroesophageal reflux is thought to contribute to development of the disease (Lee *et al.*, 2010; Richeldi, Collard and Jones, 2017). Its been shown that patients with IPF have increase oesophageal acid exposure. Another prominent study by had shown that the prevalence of acid reflux in IPF patients was 87% and significantly more common than in patients with other respiratory diseases (Raghu *et al.*, 2006).

### *Genetic Predispositions*

### *Telomerase*

A number of genetic factors have been identified in IPF development. Telomeres are a region of repetitive DNA sequences (5'-TTAGGG-3') at the end of chromosomes. They protect chromosome ends from erosion and damage. Normal DNA polymerase cannot fully replicate chromosomes to their distal ends- this is known as the end replication problem. This leads to the shortening of the chromosome, and the chromosome has reached its critical length. Once the critical length is reached the cell undergoes senescence and induces apoptosis. The number of times a cell divides before reaching the critical length and undergoes senescence is called the Hayflick Limit (Shay and Wright, 2011).

Telomerase restores the length of telomeres by adding telomeric DNA repeats at the end of chromosomes (Shay and Wright, 2011). The enzyme consists of two major components- telomerase reverse transcriptase (TERT) and telomerase RNA (TERC) (Shay and Wright, 2011). Most somatic cells do not express or express telomerase at very low levels (Hornsby, 2007). However, cancer cells express TERT at high levels ultimately leading to telomere elongation and cell immortality (Shay and Wright, 2011).

Family based studies have shown a link between fibrosis and the shortening of telomeres (Stuart *et al.*, 2014, 2015). Telomere lengths have been shown to be shorter in IPF patients compared with controls (Stuart *et al.*, 2014, 2015).

Additionally, telomere lengths can be used as a predictor of survival for patients with IPF (Stuart *et al.*, 2014, 2015). Defects in telomerase, through mutations to TERT and TERC have been described in cases of patients with IPF (Stuart *et al.*, 2014, 2015). Moreover, variants in genes involved in maintaining the structure of telomeres and telomerase (RTEL1 and PARN) have been associated with familial pulmonary fibrosis (Stuart *et al.*, 2014, 2015). Studies have also shown that mutations of genes involved in the biogenesis of telomerase or the integrity of telomere ends are linked to pulmonary fibrosis development (Stuart *et al.*, 2014, 2015).

### *MUC5B*

A common polymorphism associated to IPF is the mutation to the mucin 5b (MUC5B) gene (Seibold *et al.*, 2011). The mutation associated with this is found of the single-nucleotide polymorphism on the rs35705950 on the promotor region of the gene encoding MUC5B (Seibold *et al.*, 2011). MUC5B encodes for the protein mucin 5b (Seibold *et al.*, 2011). Mucins are a large family of glycoproteins expressed by the epithelia of the respiratory tract (Lillehoj *et al.*, 2013). They are classified as either membrane bound mucins or secretory mucins (Lillehoj *et al.*, 2013). Secretary mucins are released by club cells in the lungs and play a pivotal role in the innate defence (Lillehoj *et al.*, 2013). They coat the epithelial surface to form a protective barrier (Lillehoj *et al.*, 2013). Membrane bound mucins are generally localised to the apical surfaces of epithelial cells and participate in transduction events (Lillehoj *et al.*, 2013).

Secreted mucins form mucus in the extracellular spaces, around the epithelial layer that is densely populated with ciliated cells (Lillehoj *et al.*, 2013). Mucus is comprised of water, salt, and cellular debris all held together by mucin glycoproteins. In the protection against inhaled agents, mucus together with cilia play an important role. Disruptions to the mucins produced ultimately impacts the muco-ciliary clearance mechanism, leading to the impaired ability to trap or remove harmful inhaled particles (Lillehoj *et al.*, 2013). Accordingly, Molyneaux and colleges had shown that patients with IPF had a higher bacterial burden compared to control which was associated with the polymorphism to the MUC5B gene (Molyneaux *et al.*, 2014).

The polymorphism of rs35705950 on the MUC5B gene results in excessive MUC5B production (Seibold *et al.*, 2011). This could lead to impaired muco-ciliary clearance in the airways (Seibold *et al.*, 2011). As there is defective ciliary clearance the clearance of harmful particles such as bacteria is impaired, leading to chronic infection, inflammation, and lung remodelling. The expression of MUC5B in the lungs is increased in patients with IPF compared with controls (Seibold *et al.*, 2011). Furthermore, it has been shown that honeycomb cysts, characteristic of pulmonary fibrosis, contain increased MUC5B protein (Seibold *et al.*, 2011).

The rs35705950 polymorphism has led to the hypothesises of its involvement in the IPF disease mechanism (Seibold *et al.*, 2011). It is postulated that excessive

production of MUC5B by alveolar epithelial precursor cells in response to regenerating the injured epithelium results in an impaired repair pathway (Seibold *et al.*, 2011; Evans *et al.*, 2016). This ultimately leads to fibroblast proliferation and honeycomb cyst formation. Another hypothesis regarding the MUC5B polymorphism is that IPF is caused by a defect in the muco-ciliary clearance mechanism caused by increased MUC5B release (Seibold *et al.*, 2011; Evans *et al.*, 2016). This leads to retention of particles that cause the recurrent injury to the epithelial cells and inflammation (Evans *et al.*, 2016). Over time, the persistent lung injury leads to increased fibroblast activity and the formation of fibroblastic foci (Evans *et al.*, 2016).

### *AKAP13*

A recent genome-wide association study (GWAS) conducted by Allen *et al*. (Allen *et al.*, 2017) had found a significant variant of the rs62025270 allele near A-kinase anchoring protein 13 (AKAP13) associated with IPF susceptibility. In this largest IPF genetic study to date, they compared genomic data from 2760 patients with IPF and 8561 controls and found a significant association of the single-nucleotide polymorphism (SNP) at rs62025270 that associated with increased susceptibility of IPF and thought to be a potential driver of IPF pathogenesis. The SNP rs62025270 led to increased expression of AKAP13 in the lungs (Allen *et al.*, 2017). Increased AKAP13 expression was primarily found in the epithelial cells and lymphoid tissue, and expression was increased in fibrotic lung tissue compared to healthy control, as determined by immunohistochemistry (Allen *et al.*, 2017).

Findings from this GWAS study support the hypothesis that IPF is a disease characterised by epithelial injury. AKAP13, highly expressed by epithelial cells, is a RhoA guanine nucleotide exchange factor (RhoGEF). It coordinates thrombin and lysophosphatidic acid (LPA) receptor signalling. Thrombin and LPA signalling have been identified as profibrotic signalling pathways acting via G-protein coupled receptors and RhoA signalling pathways in epithelial cells to promote αvβ6 integrin mediated TGFβ activation (Jenkins *et al.*, 2006; Xu *et al.*, 2009). Thus, it is thought AKAP13 promotes IPF through regulating RhoA activity and promoting αvβ6 integrin mediated TGFβ activation in response to epithelial injury.

## IPF treatments

As yet, there is no cure for IPF, nor is there a treatment that can reverse the fibrosis. Currently there are only two licenced pharmacological treatments used to manage IPF. Pirfenidine and Nintedanib can only slow down the progression of the fibrosis (Azuma *et al.*, 2010; Taniguchi *et al.*, 2010; Noble *et al.*, 2011, 2016a; Richeldi *et al.*, 2011; King *et al.*, 2014). The only "cure" for IPF is lung transplant.

### *Management*

Although current treatments are non-curative, the disease can be managed to ease symptoms. If patients are smokers, they are encouraged to quit smoking.

Oxygen can also be prescribed to patients with hypoxemia at rest or with oxygen saturation less than 88%. This is to ease the symptom of breathlessness.

### *Lung transplant*

Lung transplant is the only therapy that can prolong survival in advance IPF (King, Pardo and Selman, 2011). Though lung transplantation can prolong survival and improve quality of life for the patients, around 66% of transplant patients with IPF survive for more than 3 years (Singer *et al.*, 2017; Valapour *et al.*, 2017a). 5 years post-transplantation survival in patients with IPF is seen to be around 44% (King, Pardo and Selman, 2011). Common complications related to lung transplant include infections, cancer, allograft rejection (Valapour *et al.*, 2017b). Unfortunately, most patients are not eligible for lung transplantation due to old age or complicated medical conditions.

### *Pirfenidone*

Pirfenidone was the first drug to be licensed for IPF treatment, yet its mechanism of action is not yet fully understood. Although it is thought to exert anti-fibrotic and anti-inflammatory effects through multiple pathways leading to the suppression of fibroblast proliferation and migration, and reductions in growth factors such as TGFβ, PDGF and basic fibroblast growth factor (bFGF) (Oku *et al.*, 2008; Lehtonen *et al.*, 2016a). Studies using animal models have shown that

Pirfenidone supresses the infiltration of inflammatory cells, and decreases inflammatory cytokines such as transforming necrosis factor alpha (TNFα) (Oku *et al.*, 2008). *In vitro*, Pirfenidone has shown to inhibit fibroblast proliferation and collagen synthesis (Hewitson *et al.*, 2001; Lehtonen *et al.*, 2016b). *In vivo,* Pirfenidone attenuated bleomycin induce lung fibrosis in hamsters (Iyer *et al.*, 1995; Iyer, Gurujeyalakshmi and Giri, 1999; Iyer, Hyde and Giri, 2000). This was attributed to Pirfenidone reducing bleomycin induced influx of inflammatory cells and also reducing bleomycin induced TGFβ activity (Iyer, Gurujeyalakshmi and Giri, 1999; Iyer, Hyde and Giri, 2000). However, the precise mechanism of action of Pirfenidone is still unknown.

The SP3 trial which evaluated Pirfenidone which was conducted in Japan (Taniguchi *et al.*, 2010), treatment with Pirfenidine significantly reduced the decline in the vital capacity (VC) in patients compared to placebo after 52 weeks of treatment. The PIPF-004 trail had shown that Pirfenidine significantly reduced the decline in FVC compared to placebo after 72 weeks of treatment (Noble *et al.*, 2011). However, this was not seen in a similar trail called PIPF-006 (Noble *et al.*, 2011). There have been numerous adverse effects associated with Pirfenidine treatment, such as photosensitivity, and gastrointestinal related symptoms such as diarrhoea, nausea, and dyspepsia (Azuma *et al.*, 2010; Taniguchi *et al.*, 2010). The CAPACITY trial (King *et al.*, 2014), which lead to FDA registration of pirfenidone in the USA showed that Pirfenidone treatment decreased disease progression. Furthermore, analysis of data pooled from three phase 3 clinical trials had shown that Pirfenidone reduced the

proportion of patients with more than 10% decline in FVC by 43% (Noble *et al.*, 2016b).

### *Nintedanib*

Nintedanib is a tyrosine kinase inhibitor and targets the growth factors PDGF, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) (Wollin *et al.*, 2014). It is thought to have its anti-fibrotic effects through reducing inflammatory cell infiltration, fibroblast proliferation, and fibroblast transformation to myofibroblasts (Wollin *et al.*, 2014, 2015a). *In vivo* data had shown that Nintedanib decreased bleomycin induced lung fibrosis in mice (Chaudhary *et al.*, 2007; Wollin *et al.*, 2014, 2015b). Recently it has been shown that Nintedanib reduced both fibronectin and collagen expression, induces autophagy, and inhibits TGFβ signalling in IPF fibroblasts (Robert Guzy *et al.*, 2017). Data from clinical trials had shown that Nintedanib reduced the decline in FVC (Richeldi *et al.*, 2011). Adverse effects reported with treatment with the drug are similar to that of the treatment with Pirfenidine: diarrhoea and nausea (Richeldi *et al.*, 2011).

Altogether, the precise mechanism of action for both the drugs are still unknown. But both drugs do slow down the progression of the disease. However, even with these treatments fibrosis, respiratory decline, and death is inevitable. Hence a drug which can completely halt, or reverse fibrosis needs to be found.

## Pathogenesis of IPF

The precise mechanism of IPF development and progression is still unknown (Meltzer and Noble, 2008; Martinez *et al.*, 2017). The current hypothesis of IPF development describes development of IPF through repetitive subclinical injury to the alveolar epithelium (Meltzer and Noble, 2008; Martinez *et al.*, 2017). These repetitive injuries subsequently disrupt the alveolar-capillary basement membrane (Meltzer and Noble, 2008; Martinez *et al.*, 2017). In a genetically susceptible and aged individual there is a dysregulated epithelial repair response (Meltzer and Noble, 2008; Martinez *et al.*, 2017). This failure in the alveolar epithelia to repair arises from increased epithelial cell apoptosis, and premature epithelial cell senescence (Meltzer and Noble, 2008; Martinez *et al.*, 2017). Coupled with the inability of the injured epithelia to repair itself and the infiltration of fibroblasts and myofibroblasts that synthesise high quantities of extracellular matrix proteins (ECM), which deposit and accumulate, leading to the development of fibrosis (Meltzer and Noble, 2008; Martinez *et al.*, 2017).

In the fibrotic process, the abnormal epithelial repair response gives rise to a dysregulated epithelial-mesenchymal crosstalk (Meltzer and Noble, 2008; Martinez *et al.*, 2017). The mechanism of this dysregulated epithelial-mesenchymal crosstalk is considered to be due to pro-fibrotic cytokines such as TGFβ and PDGF (Meltzer and Noble, 2008; Martinez *et al.*, 2017). These pro-fibrotic cytokines fuel this abnormal

repair response and the release and accumulation of ECM proteins leading to fibrosis (Meltzer and Noble, 2008; Martinez *et al.*, 2017).

### *Role of the epithelia in IPF*

The epithelial layer in the airways is a pseudostratified mucosal barrier consisting of multiple cell types. The epithelium serves as the first barrier in the defence against environmental particles and infectious pathogens. As well as acting as a physical barrier, the epithelium also initiates the innate and adaptive immune system to counter pathogens.

In normal wound healing following injury the epithelium and tissue architecture is rapidly restored to maintain normal gas exchange. The process is spatially and temporally regulated through controlled inflammatory and coagulation pathway responses as well as the formation of a temporary matrix. A basal layer is formed from myofibroblasts migrating to the matrix. The myofibroblasts release ECM proteins which deposit and which forms a provisional matrix. Epithelial cells regenerate (reepithelialisation) the damaged area of lung through the release of certain cytokines and growth factors. The cellular debris and provisional matrix are then removed. Reepithelialisation to restore the basement membrane integrity occurs through epithelial cell migration, proliferation, and differentiation.

Gaseous exchange takes place in the alveolus. It is lined by two types of epithelial cells. Type 1 alveolar epithelial cells (AECs) cover most of the airway surface and promote gaseous interchange. Type 2 AECs secrete surfactant proteins to aid in the gas exchange process (Camelo *et al.*, 2014). In the normal wound repair process, type 2 pneumocytes serve as the progenitor cells for type 1 AECs. Type 2 AECs are more resistant than type 1 AECs to injury and therefore regenerate the damaged epithelial. They migrate to the injured area, differentiate into type 1 AECs, and proliferate to re-establish the alveolar epithelial layer.

The epithelial lining of the alveolus is also populated with numerous other cells. Club cells are the bronchiolar secretory cells, and ciliated and basal cells are also found in the alveolar epithelium. The secretory cells release mucus into the apical surface of the epithelium to trap micro-organisms, air pollutants, and inhaled pathogens. Ciliated cells then work in synergy with goblet cells to clear out the trapped particles by beating in a rhythmic pattern to move the particles out.

The molecular mechanisms of IPF is still not completely understood. Initially it was thought that IPF was caused by chronic inflammation, but the theory now is that the disease is caused by alveolar epithelial injury followed by abnormal wound healing responses leading to atypical reepithelization and repair (Martinez *et al.*, 2017). Leading to increases in profibrogenic mediator production (Martinez *et al.*, 2017). The causes of the alveolar epithelial injury are still unknown. increased epithelial cell apoptosis and senescence are thought to be critical factors in the

abnormal epithelial cells repair response. This feature is also seen in the bleomycin lung fibrosis model, where increased epithelial cell apoptosis is seen in the development of the disease (Kuwano *et al.*, 1999). The alveolar epithelial cell injury leads to the loss in integrity of the alveolar capillary basement membrane (Selman, King and Pardo, 2001; Martinez *et al.*, 2017). This subsequently leads to the infiltration of fibrogenic and inflammatory cells which release profibrotic growth factors and mediators resulting excess abnormal matrix synthesis and accumulation.

The ECM accumulation replaces the normal alveolar structures (Type 1 and 2 AECs). Studies of IPF lung tissue and epithelial cells isolated from IPF patients have shown significant changes (Seibold *et al.*, 2013). These studies have shown mutations affecting AEC type 2 functions- surfactant protein mutations (Nogee *et al.*, 2001; Wang *et al.*, 2009; Whitsett, Wert and Weaver, 2015).

Injury leads to the alveolar epithelial cells to secrete and activate profibrotic mediators such as TGFβ and PDGF (Khalil *et al.*, 1996; Selman, King and Pardo, 2001; Martinez *et al.*, 2017). The release of these growth factors promotes the proliferation and migration of fibroblasts. Through the disease process, fibroblasts change from a migratory phenotype to a proliferative phenotype and lastly a profibrotic phenotype that produce ECM proteins. The excess synthesis and deposition of ECM proteins leads to fibrosis and remodelling of the lungs leading to the distinct honeycomb appearance characterised in IPF. Excess release of ECM proteins is attributed to the increased activation and activity of myofibroblasts (Selman *et al.*, 1986; Selman, King

and Pardo, 2001; Martinez *et al.*, 2017). Increases in myofibroblasts is characteristic in the pathology of IPF where fibroblastic foci are a key feature in UIP (Gross and Hunninghake, 2001; Raghu *et al.*, 2018). This together with the failure of epithelial repair, increased epithelial cell apoptosis, and decreased epithelial cell proliferation promotes fibrosis (Gross and Hunninghake, 2001; Selman, King and Pardo, 2001; Martinez *et al.*, 2017).

### *Role of Fibroblasts in IPF*

Fibroblasts are resident mesenchymal cells that are thought to originate from embryonic mesoderm tissues (Lynch and Watt, 2018). They are found in the interstitial spaces of all organs. Once differentiated, they synthesise and release structural ECM proteins such as collagen to provide a structural framework for tissues (Burgstaller *et al.*, 2017). They do not express alpha smooth muscle actin ($\alpha$SMA), and lack contractile microfilamentous apparatus (stress fibres) that are observed in myofibroblasts (Phan, 2008). These cells however do express vimentin and type 1 collagen, which are conventionally considered fibroblast markers (Phan, 2008). The heterogeneous nature of fibroblasts depends on the stage of development, tissue of origin, and the tissue microenvironment (Phan, 2008).

Fibroblasts play a critical role in wound healing. Fibroblast activation is vital role in the wound healing response. However, in disease they play a central role, where they release pro-fibrotic mediators to induced chronic inflammation and

excessive ECM production and accumulation (Phan, 2008). In normal wound healing, fibroblasts are activated, proliferate, and migrate to the injured site. They then differentiate into myofibroblasts, synthesise the ECM proteins creating a provisional matrix structure to help with tissue regeneration (Phan, 2008). In addition, fibroblasts produce matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in the final stages of the remodelling process (Phan, 2008). The MMPs degrade the provisional matrix once the wound healing process is complete and the myofibroblasts are removed. Dysregulation to this process results in ineffective matrix degradation and continued ECM release and accumulation. Fibrotic fibroblasts are also seen to be resistant to apoptosis (Tanaka *et al.*, 2002).

In IPF, there is a dysregulation in this wound healing process. The repeated damage of the alveolar epithelium results in epithelial damage and apoptosis. In response cytokines and growth factors released, fibroblasts are recruited and migrate to the injured site (Quesnel *et al.*, 2010; Tschumperlin, 2013; Prasad, Hogaboam and Jarai, 2014). The accumulation of fibroblasts and myofibroblasts generate fibroblastic foci, which are hallmarks of the disease (Martinez *et al.*, 2017). It has been seen that increase number of fibroblastic foci is associated with disease progression and worsened prognosis (Martinez *et al.*, 2017).

### *Myofibroblasts*

The major effector cell in the fibrotic process is the myofibroblasts (Meltzer and Noble, 2008; Martinez *et al.*, 2017). These cells drive the progression of fibrosis. They are the primary source of fibrotic ECM and lead to structural remodelling (Burgstaller *et al.*, 2017). Myofibroblasts are highly contractile and are characterised by expression of α-smooth muscle actin (αSMA) and other stress fibres (Phan, 2002, 2008). Hence, they share features of both smooth muscle cells and fibroblasts. The contractile apparatus that characterises myofibroblasts contains bundles of actin microfilaments and contractile proteins (Burridge and Chrzanowska-Wodnicka, 1996; Phan, 2008). The actin structures terminate at the surface of the myofibroblast at the fibronexus. The fibronexus is a large complex composed of integrins and links intracellular actin to the extracellular fibronectin fibrils (Burridge and Chrzanowska-Wodnicka, 1996; Dugina *et al.*, 2001; Phan, 2008). Overall this structure enables myofibroblasts to provide a mechano-transduction system, where the force generated by the stress fibres can be transmitted to the ECM (Burridge and Chrzanowska-Wodnicka, 1996; Phan, 2008). Additionally, in this mechano-transduction system the extracellular mechanical signals can be transduced into intracellular signals (Burridge and Chrzanowska-Wodnicka, 1996).

The expression of αSMA and collagen in myofibroblasts is regulated by growth factors such as TGFβ (Desmoulière *et al.*, 1993; Hinz, 2007). Released mediators such as particular growth factors and cytokines cause the differentiation of certain cells into myofibroblasts (Micallef *et al.*, 2012). The TGFβ that is found in the damaged tissues is derived from platelets, white blood cells, and parenchymal

cells (O'Kane and Ferguson, 1997; Vaughan, Howard and Tomasek, 2000; Jenkins *et al.*, 2006; Xu *et al.*, 2009). Fibroblasts also produce TGFβ to maintain the wound healing process (Kendall *et al.*, 2014). In turn, the myofibroblasts not only synthesise and release ECM protein but also release growth factors such as TGFβ to augment the fibrotic process in disease. Furthermore, in normal wound healing process the number of myofibroblasts gradually decline to complete the successful healing process. However, in IPF myofibroblasts become resistant to apoptosis.

The presence of myofibroblasts in the fibrotic lesions of experimentally induced lung fibrosis in animals is associated with only fibrosis development (K Zhang *et al.*, 1994). Myofibroblasts harvested from the lung of IPF patients have been shown to have an enhanced migratory phenotype, release greater pro-fibrotic mediators, and are more resistant to apoptosis (Suganuma *et al.*, 1995; Ramos *et al.*, 2001; Moodley *et al.*, 2003, 2004). Additionally, it has been associated with disease progression in humans (Kuhn and McDonald, 1991).

The contractile force generated by myofibroblasts is due to the contractile stress fibres found in the cell's cytoskeleton (Phan, 2008). The force is regulated by myosin light-chain (MLC) phosphorylation, which is similar to what is seen in smooth muscle cells (Chrzanowska-Wodnicka and Burridge, 1996). The phosphorylation of the MLC is regulated by two systems- calcium-dependant MLC kinase and the Rho kinase pathway (Burridge and Chrzanowska-Wodnicka, 1996). Increases in the intracellular calcium levels activate the MLC kinase pathway. This leads to the

phosphorylation of MLC and triggering myofibroblast contraction (Burridge and Chrzanowska-Wodnicka, 1996). Within, the Rho kinase pathway the small Rho GTPase is activated. Consequently, activating downstream Rho effectors found within the signalling pathway such as Rho kinase 1 and 2 (ROCK1 and 2). Subsequently, this leads to increase MLC phosphorylation and increased tension. It has been noted that there are two pathways by which Rho kinases promote MLC phosphorylation. They can directly phosphorylate the MLC, and they can directly inhibit the activity of myosin phosphatase by phosphorylating the myosin subunit of the enzyme (Kimura *et al.*, 1996; Kawano *et al.*, 1999).

The origins of myofibroblasts are controversial. However, it is though myofibroblasts arises from three main sources; from resident parenchymal fibroblasts which transdifferentiate into myofibroblasts, circulating precursor cells such as fibrocytes, and from lung epithelial cells which differentiate to myofibroblasts through a process known as epithelial to mesenchymal cell transition (EMT) (Zhang *et al.*, 1994). In responses to growth factors such as TGFβ, PDGF, and connective tissue growth factor (CTGF), quiescent resident parenchymal fibroblasts are activated, proliferate and differentiate into myofibroblasts (Leask and Abraham, 2003; Bonner, 2004; Scotton and Chambers, 2007). The precise origin of the myofibroblasts that arise from differentiated fibroblasts in IPF is unknown, but it thought that they are derived from parabronchial and perivascular fibroblasts (Zhang *et al.*, 1996).

Fibroblasts in IPF further contribute to fibrosis through activation of pro-survival pathways enabling the fibroblasts to become resistant to apoptosis (Chambers, 2008; Vuga *et al.*, 2009; Chang *et al.*, 2010). Thus, in IPF fibroblasts accumulate augmenting ECM release and deposition, resulting in fibrosis. Myofibroblast activation is summarized in figure 1.1.

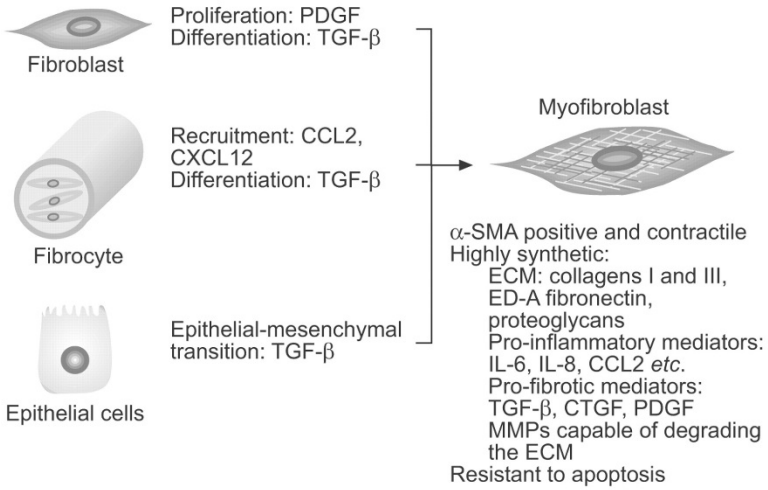



*Figure 1.1*

Myofibroblasts are play a key role in the pathogenesis of IPF. Myofibroblasts release large amounts of ECM proteins such as collagen which deposit and accumulate leading to fibrosis. Myofibroblasts arise from 3 main sources: fibroblasts, fibrocytes, and epithelial cells through EMT. Figure adapted from paper by Chambers (Chambers, 2008).

### *Fibrocytes*

Fibrocytes are bone-derived circulating fibroblast progenitor cells aid in wound healing and the fibrosis process. They are attracted to injured tissues through the signalling of chemokines. Recent and increasing evidence has been suggesting that bone marrow derived and tissue resident progenitor cells play a role in the injury repair response in the lungs (Moore *et al.*, 2006; Beers and Morrisey, 2011; Mehrad

and Strieter, 2012). In IPF, it is thought that there in increased recruitment of mesenchymal progenitor cells leads to the exaggerated repair response.

Circulating in the peripheral blood, bone marrow derived fibrocytes contribute to fibrosis through differentiating into the ECM producing myofibroblasts (Bucala *et al.*, 1994; Hong *et al.*, 2007; El-Asrar *et al.*, 2008). Fibrocytes are also able to produce collagen and other ECM proteins, cross linking enzymes, and growth factors and cytokines. They express mesenchymal markers such as type 1 collagen and leukocyte marker CD45 (Kleaveland *et al.*, 2014). When cultured in vitro, these cells develop a spindle shaped morphology (Bucala *et al.*, 1994). Fibrocytes also express certain chemokine receptors such as CXCR4 and CCR7 (Abe *et al.*, 2001).

Fibrocytes respond to the profibrotic cytokine TGFβ resulting in the increase of expression of αSMA, thus causing the differentiation of fibrocytes to myofibroblasts (Hong *et al.*, 2007). Consequently, this differentiation drives the fibrotic process. There are higher levels of fibrocytes in the peripheral blood of patients with IPF indicating that fibrocytes play a vital role in pulmonary fibrosis development (Andersson-Sjöland *et al.*, 2008). In addition, fibrocytes are thought to be the source of fibroblasts that create the fibroblastic foci.

In animal models it has been shown that inhibiting migration of fibrocytes into the lungs blocks the development of pulmonary fibrosis (Moore *et al.*, 2003). Furthermore, Philips *et al.* had shown that in response to bleomycin treatment

fibrocytes migrate to the injured lung, where peak infiltration is seen at day 8 post bleomycin treatment (Phillips *et al.*, 2004). The recruitment of fibrocytes directly correlated with increased collagen deposition (Phillips *et al.*, 2004). Additionally, by blocking CXCL12, fibrocyte recruitment was decreased leading to an attenuated bleomycin induced lung fibrosis (Phillips *et al.*, 2004).

### *Epithelial to mesenchymal cell transition (EMT)*

Epithelial cells transform into matrix producing myofibroblasts through a process called EMT (Moustakas and Heldin, 2007). EMT is characterised by the loss of epithelial markers such as E-Cadherin and the acquisition of the mesenchymal markers αSMA, vimentin, and collagen 1 (Kasai *et al.*, 2005). EMT is thought to be involved in the pathogenesis of numerous lung diseases such as lung fibrosis and lung cancer. Additionally, EMT has been reported in various organs such as the kidneys and liver. The EMT process has been shown to be important in the embryonic development, where the process plays roles in the development of the neural crest and organs.

It is thought that in pulmonary fibrosis around 30% of fibroblasts are derived from epithelial cells (Kim *et al.*, 2006). TGFβ has been shown to be a key inducer of EMT, where it has been shown to drive EMT *in vitro, ex vivo,* and *in vivo*. (Kasai *et al.*, 2005; Kim *et al.*, 2006). Immunofluorescent staining in human IPF lungs had shown that cells within the remodelling matrices contain both epithelial and mesenchymal

cells, suggesting these are areas of active EMT (Kim *et al.*, 2006). Mice with lung epithelial cell specific loss of the integrin subunit $\alpha 3$ expression had an attenuated response to bleomycin induced lung injury and markedly impaired EMT (Kim *et al.*, 2006). EMT of alveolar epithelial cells has been observed in IPF patients (Kim *et al.*, 2006; Marmai *et al.*, 2011; Bartis *et al.*, 2014; O'Beirne *et al.*, 2015).

## *The role of the extracellular matrix in IPF*

### *MMPs*

In the normal remodelling process, the temporary matrix is removed. The removal is largely mediated by a group of zinc dependent endopeptidases known as MMPs. MMPs are a group of enzymes involved in the degradation of the ECM (Lu *et al.*, 2011). Currently there are 24 known MMPs, and the MMP family can be divided into seven groups: membrane type MMPs, interstitial collagenase, stromelysins, gelatinase, matrilysins, metalloelastase, and other non-classified MMPs (Lu *et al.*, 2011). Recent evidence has shown that MMPs are not only involved in ECM turnover but also in inflammation, promotion of pro-fibrotic growth factors, and the cleavage of receptors and cell surface molecules (Craig *et al.*, 2015). Tissue inhibitors of MMPs (TIMPs) serve as endogenous inhibitors of MMPs. TIMPs work by binding to the active site of MMPs (Nagase, Visse and Murphy, 2006).

MMPs are synthesised as latent enzymes that require activation to exert their catalytic activity. They can be activated by proteinases and reactive oxygen species (Craig *et al.*, 2015). It is commonly thought that IPF is a disease caused by attenuated ECM degradation and increased deposition. However, it is been found that some of the MMPs are highly expressed in the lungs and blood from patients with IPF compared to control (Craig *et al.*, 2015; Pardo *et al.*, 2016). *In vivo* studies using specific MMP knock-out mice have shown the knock-out of certain MMPs (e.g. MMP3, 7, 8) protected mice from bleomycin induced lung fibrosis (Craig *et al.*, 2015; Pardo *et al.*, 2016).

MMP3 is expressed by epithelial cells, fibroblasts and macrophages; and has been shown to cleave type IV collagen in vitro (N Kaminski *et al.*, 2000; Yamashita *et al.*, 2011). The expression of MMP3 is upregulated in patients with IPF, where increases were found in the alveolar epithelial cells, fibroblasts, and macrophages (Yamashita *et al.*, 2011). MMP3 knock-out were protect against bleomycin induced lung fibrosis (Yamashita *et al.*, 2011). The pro-fibrotic nature of MMP3 was shown by stimulating epithelial cells with MMP3, which resulted in the activation of EMT pathways through the β-catenin pathway (Yamashita *et al.*, 2011). MMP3 has also been shown to promote the activation of TGFβ by releasing the latent TGFβ molecule from the latent TGFβ complex (Maeda *et al.*, 2002).

Likewise, MMP7 has also been shown to mediate pro-fibrotic effects (Pardo *et al.*, 2005). MMP7 levels are increased in the plasma and BALF of patients with IPF

(Rosas *et al.*, 2008). MMP7 null mice are protected from bleomycin induced lung fibrosis, demonstrating the potential pro-fibrotic role of MMP7 (Zuo *et al.*, 2002). Accordingly, MMP7 may promote fibrosis by mediating the shedding of the E-cadherin ectodomain in epithelial cells leading to the failure of epithelial repair (McGuire, Li and Parks, 2003).

### *ECM proteins*

The lung provides the mechanical stability, support, and the elastic recoil that are imperative for lung normal function. The ECM also plays an essential role in lung homeostasis, remodelling, and development by providing biochemical and biomechanical signals to effect cell function and differentiation (Burgstaller *et al.*, 2017). Proteins that comprise the ECM include collagens, elastin, glycosaminoglycans, and laminins (Burgstaller *et al.*, 2017). Furthermore, proteomic studies have revealed that the ECM contains over 150 different proteins, glycosaminoglycans, and enzymes (Burgstaller *et al.*, 2017). Lung resident fibroblasts have been identified at the major source of ECM production.

In patients with IPF, procoagulant factors such as fibrinogen, thrombin, and factors VII and X are increased (Peyrol, Cordier and Grimaud, 1990). Thrombin in particular is able to increase fibroblast proliferation, collagen synthesis, and fibroblast to myofibroblast differentiation (Chen and Buchanan, 1975; Bogatkevich *et al.*, 2001; Chambers *et al.*, 2015). These factors are increased in the normal wound

healing process to create the provisional ECM. This process is controlled in healthy individuals, however in disease there is a loss in this regulation leading to increased ECM formation and deposition and decreased matrix turnover.

The pulmonary ECM is organised into 2 main structural types. First, the basement membrane which is composed of glycoproteins which line the basal side of the epithelia, endothelia, and surround the muscles and fat. Second, the interstitial matrices, which form a fibril meshwork that interconnects cells within the tissue to creates the biomechanical characteristics of the lungs.

A large portion of the ECM comprises of collagen (Suki *et al.*, 2005). The fibrillar collagens- collagen type I, II, III, V, and XI have low elasticity but a high tensile strength which contribute to the lung architecture (Suki and Bates, 2008; Bora, Faffe and Zin, 2009). Contrastingly, the elastic fibres are characterised by low tensile strength and high elasticity provide the lung with compliance and elastic recoil (Burgstaller *et al.*, 2017). Elastin in the ECM is found in its crosslinked form in the inner core of the elastic fibres. Elastin is also found on the periphery of the elastic fibres which also contain microfibrils (Wagenseil and Mecham, 2007). The interstitial ECM of the alveoli largely comprises of collagen type I and III, and elastin to form mesh-like structure (Bosman and Stamenkovic, 2003). Posttranslational modification of the ECM affects the structural and functional features of the proteins found in the ECM (Burgstaller *et al.*, 2017). An example of this being achieved is through enzyme

mediated crosslinking of collagen and elastin by members of the lysyl oxidase family (LOX 1-4) and transglutaminase family (TG1-7).

The ECM also functions as a store of a number of growth factors and cytokines such as TGFβ (Yue, 2014). TGFβ is found in a latent state, anchored to the ECM where it is stored. It therefore needs to be activated to exert its biological effect (Annes, 2003; Justin P. Annes *et al.*, 2004). Thus, the ECM is able to regulate TGFβ activity temporally and spatially.

The fibrotic ECM constitutes of collagen such as collagen type 1. The collagen accumulation in disease resulting in increased matrix stiffness is from a consequence of increased collagen synthesis and release from myofibroblasts, increases in profibrotic cytokines such as TGFβ, decrease in MMP activity, and increased collagen cross-linking that strengthen the collagen fibres. In the collagen fibril, the collagen molecules are stabilised by covalent intermolecular crosslinking (Shoulders and Raines, 2009). The crosslinking occurs between the helical and telopeptide domains on neighbouring collagen molecules. The interaction occurs between lysine, 5-hydroxylysine, and histidine residues (Shoulders and Raines, 2009). Defects in the hydroxylation of lysine result in atypical collagen crosslinking leading to bone and connective tissue disorders (Yamauchi *et al.*, 2018).

Fibronectin is another key component of the ECM. Fibronectin is released by fibroblasts (Torr *et al.*, 2015). It serves as an ECM scaffold, and has the ability to bind

to inflammatory cells and fibroblasts through certain adhesion molecules and it also orchestrates cell signalling through integrins during tissue remodelling. Fibronectin also serves as a store for latent TGFβ, where the latent TGFβ is directly coupled to the fibronectin matrix. It has been shown that stretching the ECM activates latent TGFβ bound to fibronectin (Wipff *et al.*, 2007). Additionally, conformational changes in the fibronectin matrix drive altered integrin binding leading to EMT (Markowski, Brown and Barker, 2012; Brown *et al.*, 2013).

*In vivo*, cells are constantly under strain forces and stress. In the alveolus, epithelial and fibroblasts are exposed to stress during the respiratory cycle (Waters, Roan and Navajas, 2012; White, 2015). This stress can be elevated during increases in lung stiffness which arises through increases in ECM deposition during the remodelling process and rises in cross-linking enzyme activity. Tissue stiffness is measured as Young's elastic modulus, which is defined as the degree of stress over the degree of strain and describes the objects resistance to being deformed elastically (White, 2015). Increased ECM deposition by fibroblasts and decreased ECM turnover leads to the increases in matrix stiffness. Furthermore, increased activity from cross-linking enzymes such as the LOX enzymes contribute to increases in tissue stiffness by strengthening protein-protein interactions.

The LOX enzymes catalyse the crosslinking of collagens in the extracellular spaces. They work by catalysing the oxidative deamination of lysine and 5-hydroxylysine residues in the telopeptide domains and generating reactive aldehydes

(Yamauchi and Sricholpech, 2012). The reactive aldehydes then undergo a series of condensation reactions with lysine, 5-hydroxylysine, and histidine residues to form the intramolecular crosslinks between collagen molecules (Yamauchi and Sricholpech, 2012). The lysyl oxidase-like family of enzymes (LOXL1-4) are expressed by fibroblasts (Aumiller *et al.*, 2017). In turn, the inhibition of the LOXL enzymes in fibroblasts downregulated the genes involved in fibroblast to myofibroblast differentiation (Mižíková *et al.*, 2017). Additionally, LOX is able to bind to TGFβ and supress its signalling (Atsawasuwan *et al.*, 2008).

LOXL enzymes are released by myofibroblasts and are found in the fibroblastic foci and regions of active fibrosis (Bellaye *et al.*, 2018). *In vivo* studies have shown the knock-out of LOXL2 protects mice from bleomycin induced lung fibrosis (Barry-Hamilton *et al.*, 2010). In IPF, the expression of LOXL2 and TG2 has shown to be increased in the lung and serum of patients with IPF, and accordingly high serum levels of LOXL2 has been associated with higher disease activity, progression, and mortality (Chien *et al.*, 2014). Despite promising pre-clinical data showing inhibition of LOXL2 reduces lung fibrosis in mouse models, phase 2 clinical trial of the LOXL2 inhibitor simtuzumab did not show any improvement in progression free survival in patients with IPF (Raghu *et al.*, 2017). Recently it has been shown that trihydroxyphenolic compounds can block TGFβ induced collagen production in fibroblasts by being metabolised by LOXL2 to produce an unknown compound which can directly block TGFβ receptors (Wei *et al.*, 2017).

It is thought that the ECM plays a pivotal role in driving fibrosis and the progressiveness seen in IPF. In the fibrotic matrix there is an increase in tissue stiffness, where stiffness of the tissue increases from 0.5-1kPa to around 25-100kPa. The ECM stiffness is due to the increased collagen accumulation from myofibroblasts and increased TGFβ activity.

*In vitro* studies have shown that fibroblasts have increased proliferation when grown on stiff matrices compared to softer ones (Discher, Janmey and Wang, 2005; Liu *et al.*, 2010). Moreover, there has been data suggesting that ECM stiffness in IPF promotes fibroblast activation, fibroblast to myofibroblast differentiation, increased ECM synthesis and deposition, and down regulation of antifibrotic molecules such as cyclooxygenase 2 (Liu *et al.*, 2010). Additionally, the increased matrix stiffness activates the mechanosensitive Hippo pathway effector Yes-associated protein 1 (YAP), which in turn creates a positive feedback loop to increase ECM deposition and ultimately matrix stiffness (Liu *et al.*, 2015). The expression of YAP has been shown to be increased in pulmonary fibrosis in humans (Liu *et al.*, 2015).

The interaction between cells and the ECM is termed mechanosensing. This occurs largely though integrin-based adhesion, which transmits mechanical signals from the ECM to the cell cytoskeleton and cellular signalling pathways. Subsequently, the stiffer environment creates a higher mechanical resistance to deformation which allow cells to produce higher traction forces (Santos and Lagares, 2018). Consequently, this increases activity of focal adhesion proteins. Conversely, in softer

matrices lower traction forces are generated by cells on the ECM as there is a lower mechanical resistance. Integrins are heterodimers comprising of an $\alpha$ and $\beta$ subunit and serve as mechanical linkages. Activated integrins are bound to actin and myosin through the association with binding proteins such as talin and vinculin.

## Role of TGFβ in IPF

TGFβ is a member of the TGFβ superfamily that includes 33 members of different growth and differentiation factors, bone morphogenic proteins and other cytokines (Wu and Hill, 2009). In mammals there are 3 different isoforms of TGFβ (TGFβ1, TGFβ2, and TGFβ3). They play pivotal roles is tissue homeostasis, development, wound healing, tumorigenesis, and immune responses (Wu and Hill, 2009). TGFβ is involved in a wide range of cellular processes including cellular apoptosis, growth, differentiation, and migration (Wu and Hill, 2009). They exert these cellular effects by targeting serine/threonine receptors such as TGFβ receptor I (TGFβRI/ Alk5) and TGFβ receptor II (TGFβRII) which signal through the canonical Smad signalling pathway and through the non-canonical signalling pathways (Wu and Hill, 2009). TGFβ assembles TGFβRI and TGFβRII into a complex allowing the signal transduction (Wu and Hill, 2009). TGFβ overactivity has been linked with a number of diseases such as pulmonary fibrosis, liver cirrhosis, arthritis, cancer, and disease related to inflammation (Massagué, 2008; Coward, Saini and Jenkins, 2010; Dooley and ten Dijke, 2012; Han *et al.*, 2012).

The importance of TGFβ in development has been shown in TGFβ knockout mice. TGFβ1 null mice die within 3 weeks of birth from multiorgan inflammation (Kulkarni *et al.*, 1993). Also noted in the TGFβ1 null mice were the increased infiltration of inflammatory cells such as lymphocytes and macrophages within the heart and lungs (Kulkarni *et al.*, 1993). TGFβ2 null mice display severe disruption in tissue development, where heart defects and skeletal defects leading to mice dying at birth were observed (Sanford *et al.*, 1997). TGFβ3 null mice also have been shown to die shortly after birth where disruption in lung development was observed (Kaartinen *et al.*, 1995). The signalling of TGFβ is regulated through different factors- extracellular factors control TGFβ activation and intracellular factors modulate the downstream signalling.

The 3 TGFβ isoforms are structurally similar. TGFβ1 consists of 390 amino acids whereas TGFβ2 and TGFβ3 consist of 412 amino acids (Wu and Hill, 2009; Poniatowski *et al.*, 2015). The TGFβ ligands are secreted as inactive precursors, thus when secreted they contain a pro-region called the latency associated peptide (LAP) (Annes, 2003). They contain a C-terminal region that contains nine cysteines held together by eight disulphide bonds creating the cysteine knot within the molecule (Annes, 2003). Additionally, one disulphide bond which forms between a cysteine of one monomer and another creating a dimer (Annes, 2003). The TGFβ precursor proteins form a dimeric complex within the endoplasmic reticulum of cells and processed into mature TGFβ dimer and the cleaved LAP by furin cleavage in the trans-Golgi network (Robertson and Rifkin, 2013). The interaction between TGFβ and LAP

renders the growth factor latent. Furthermore, the TGFβ-LAP complex is linked to the latent TGFβ binding proteins (LTBPs) through covalent bonds forming the large latent TGFβ complex. Once secreted, the large latent TGFβ complex can covalently attach to the ECM. TGFβ1 and TGFβ3 both contain RGD motifs where integrins are able to bind and cause the activation/release of the TGFβ molecule.

## TGFβ activation and integrins

TGFβ is secreted in a latent state and must be activated to exert its biological effect. This allows for the extracellular concentration and TGFβ activity to be temporally and spatially regulated. Tissues contain significant quantities of latent TGFβ (Annes, 2003). The three TGFβs are synthesised as homodimeric proteins with a mass of 75kDa. LAP is cleaved from the mature TGFβ dimer in the trans-Golgi network by furin enzymes. Disulphide bonds are formed between cysteine residues of the LAP and cysteine residues in the LTBP, creating the large latent TGFβ complex (Annes, 2003). The LTBPs family all contain unique domains containing eight cysteine residues (Annes, 2003). LTBP1, LTBP3 and LTBP4 can bind to LAP, where the cysteine domains of the LTBPs form disulphide bonds with the cysteine domains of the LAP (Annes, 2003). The cysteine domains of the LTBPs also localise the LTBP complex to the ECM (Annes, 2003). Knockout of the cysteines that link the TGFβ propeptide to the LTBP in mice had shown that these mice produce a similar phenotype as seen in TGFβ knock out mice (Yoshinaga *et al.*, 2008). The mice experienced shortened life span and multiorgan inflammation (Yoshinaga *et al.*, 2008).

When synthesised and released TGFβ1 is stored within the ECM or tethered on cell surfaces (Munger and Sheppard, 2011). TGFβ1 is synthesised as a small latent complex. This consists of the active TGFβ1 that is non-covalently associated with a latency associated peptide (LAP) (Annes, 2003). In turn, the small latent complex is released as a large latent complex though its association (disulphide bond) with the LTBPs (Annes, 2003). When secreted the LTBPs attach via covalent bonds to components of the ECM or on cell surfaces, where the complex is stored (Annes, 2003). Thus, the LTBPs determine the distribution and tissue localisation of TGFβ. The latent structure of TGFβ1 is shown in figure 1.2. The importance of LTBPs have been shown in several mouse studies. Mice with LTBP1 knockout displayed defects in TGFβ signalling and cardiovascular development (Todorovic *et al.*, 2007). LTBP3 knockout mice develop skeletal defects and a reduction in Smad signalling (Dabovic *et al.*, 2002). Knockout of LTBP3 in mice has also shown to decrease TGFβ signalling altering cell proliferation and the development of emphysema (Colarossi *et al.*, 2005).

To exert its biological effect TGFβ needs to be activated. TGFβ1 is activated through the dissociation of the LAP to the LTBPs (Justin P. Annes *et al.*, 2004). This dissociation and consequent activation of TGFβ1 can be as a result of physical processes such as high temperatures, low pH, and oxidation. TGFβ can also be activated through a number of proteases such as tryptase, thrombin, plasmin, elastase, matrix metalloprotease 2 (MMP2) and 9 (MMP9) (Jenkins, 2008). Latent TGFβ activation can also be mediated by integrins such as αVβ1, αVβ3, αVβ5, αVβ8,

and αVβ6 which have been shown to play vital roles in TGFβ activation leading to fibrosis (Munger *et al.*, 1999; Justin P Annes *et al.*, 2004; Tatler *et al.*, 2011).

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits. The mammalian genome contains 18 $\alpha$ subunits and 8 $\beta$ subunits, where there are 24 different $\alpha$ and $\beta$ protein combinations found. Integrins act as bidirectional cell receptors receiving and transmitting signals from both inside and outside the plasma membrane. This is known as inside-out and outside-in signalling (Wipff and Hinz, 2008). Integrins are able to bind ECM proteins such as collagen, laminin and fibronectin. Additionally, they are also able to bind to growth factors and cell surface ligands to mediate cell adhesion. Integrin activity is regulated by controlling the cytoskeletal organisation. Integrin activity is also regulated through the dissociation of the two integrin subunits, whereby the association between the α and β subunits enables to integrin to function. Cytoskeletal reorganisation and integrin activation occurs through the activation of Ras homologous guanosine triphosphatase (Rho GTPase) (DeMali, 2003). Rho GTPase stimulate actomyosin-driven contractility (Ridley, 2015). Thus, integrin function largely depends on the connection to the cytoskeleton. The integrin cytoplasmic tails are connected to the F-actin filaments through proteins such as talin, filamin, vinculin, tensin, and paxillin (Schwartz, 2010).

The αV family of integrins are able to bind to ligands through the arginine-glycine-aspartic acid (RGD) sequence. 5 β integrin subunits contain αv (αvβ1, αvβ3,

αvβ5, αvβ6, and αvβ8). An RGD sequence is found in the LAP of TGFβ1 and TGFβ3, allowing their activation through αV integrins. The αVβ6 mediated TGFβ activation is the best characterised mechanism in TGFβ activation and signalling.

AVβ6 is an epithelial specific integrin. Its expression is increased in injured epithelium. High levels of αVβ6 have been demonstrated in the epithelium of lung tissues from IPF patients, and is associated with a worse prognosis (Xu *et al.*, 2009; Saini, Porte, Weinreb, Violette, Wallace, Mckeever, *et al.*, 2015). αVβ6 mediated TGFβ activation has been shown to be fundamental in the development of pulmonary fibrosis (Munger *et al.*, 1999; Horan *et al.*, 2008; Puthawala *et al.*, 2008).

In latent TGFβ activation the cytoplasmic tail of the β6 subunit binds to the actin cytoskeleton. On the cell surface the αVβ6 integrin binds to the RGD motif of LAP in the small latent complex, When the αVβ6 integrin is activated this leads to conformational changes in the TGFβ complex and its consequential activation (Justin P. Annes *et al.*, 2004). As the activated TGFβ is not released into the extra cellular spaces it can only interact with TGFβ receptors on adjacent cells. Accordingly, it has been shown that for αVβ6 meditated TGFβ activation to occur there needs to be cell-cell contact between the TGFβ receptor-expressing cell and the αVβ6-integrin-expressing cell (Annes, 2003). AVβ6 mediated TGFβ activation is summarised in figure 1.3. In normal lung tissues, due to the intact basement membrane the αVβ6 mediated TGFβ activation and TGFβ-TGFβ receptor interaction would be restricted to the epithelial cells (Tatler and Jenkins, 2012). However in IPF, it is proposed that

as the basement membrane is impaired the activated TGFβ is able to interact with TGFβ receptors on fibroblasts and myofibroblasts (Tatler and Jenkins, 2012). This results in the release in profibrotic mediators and ECM protein deposition leading to fibrosis. In the injured lung, TGFβ also amplifies this response through a positive feedback mechanism where activated TGFβ increases the transcription of the β6 subunit through upregulating itgb6 expression (Wang *et al.*, 1996; Tatler *et al.*, 2016). TGFβ and itgb6 feedback mechanism is thought to be mediated through the dysregulation of the Ets family of transcription factors such as Elk1 (Tatler *et al.*, 2016).

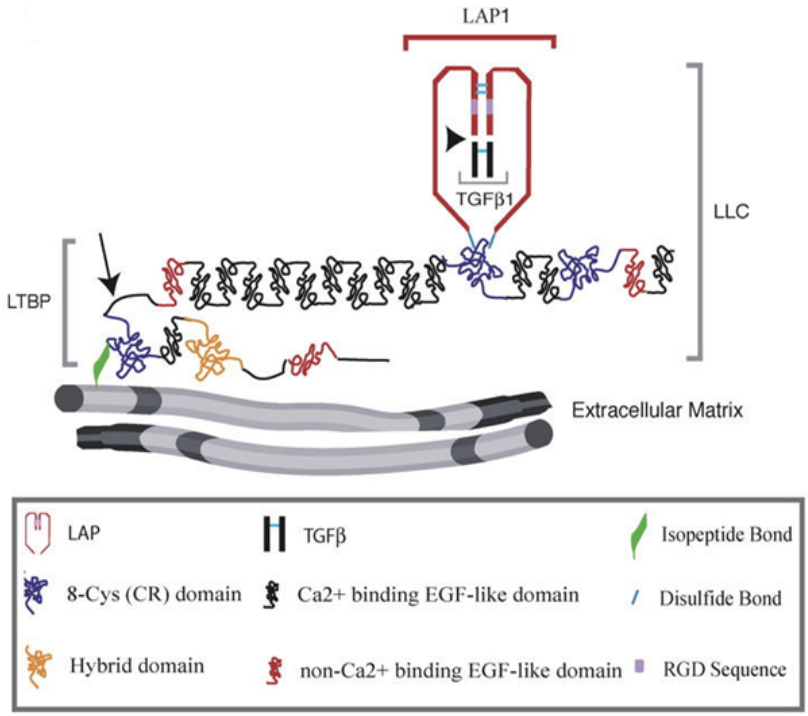



*Figure 1.2*

Latent structure of TGFβ1. TGFβ1 is synthesised as a small latent complex (SLC) which consists of active TGFβ1 attached to a latency associated peptide (LAP). The SLC in turn is released attached to TGFβ binding protein (LTBP) creating a structure known as the large latent complex (LLC). The latent TGFβ1 complex is sequestered on the ECM through attachment of the LTBP to the ECM, where it remains until the TGFβ1 is activated. Figure adapted from Annes *et al*. (Justin P. Annes *et al.*, 2004).

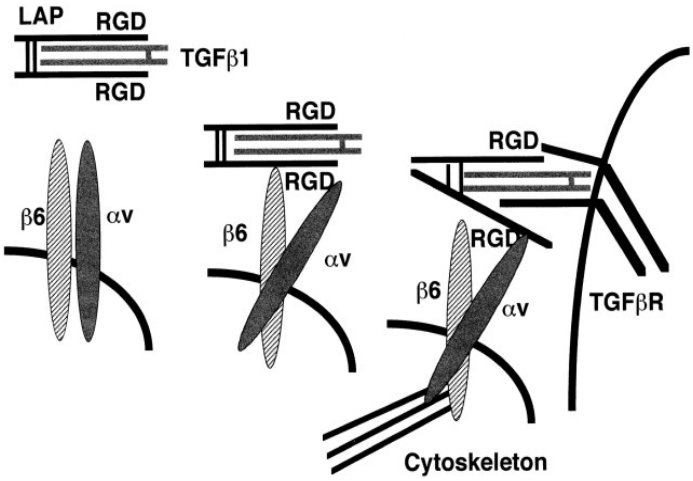



*Figure 1.3*

αVβ6 mediated TGF-β activation. Integrin αVβ6 binds to the LAP of the LLC through the RGD motif. This creates a conformational change of the latent complex allowing TGF-β to become accessible and bind to TGF-β receptors. Figure adapted from Munger *et al*. (Munger *et al.*, 1999).

## TGFβ signalling pathways

TGFβ exerts its cellular effects by activating serine/threonine receptors such as TGFβ receptor I (TGFβRI/ Alk5) and TGFβ receptor II (TGFβRII). When TGFβ Binds to TGFβRII it results in a complex formation between TGFβRII and TGFβRI (Wrana *et al.*, 1992; Anders and Leof, 1996). Consequently, this leads to the activation of a downstream signalling cascades. TGFβ primarily signals through the canonical Smad signalling pathway (Nakao *et al.*, 1997; Feng and Derynck, 2005; Hill, 2009; Macias, Martin-Malpartida and Massagué, 2015). However, non-canonical signalling pathways are also observed (Derynck and Zhang, 2003; Moustakas and Heldin, 2005).

Once phosphorylated by TGFβRI, Smad2 or Smad3 associates with Smad4. The Smad2/Smad4 or Smad3/Smad4 complex then translocates to the nucleus to regulate gene transcription. Non-canonical (non-Smad2) pathways can also be activated by TGFβ, such as PI3K/AKT (Zhang, Zhou and ten Dijke, 2013; Bauer *et al.*, 2015), MAPK/ERK (Chapnick *et al.*, 2011; Bauer *et al.*, 2015), Wnt (Piersma, Bank and Boersema, 2015), and notch pathways (Fu *et al.*, 2009).

## TGFβ activity in normal wound repair

During normal wound healing TGFβ signalling is spatially and temporally regulated; its expression and activation is increased during repair to promote the synthesis of the ECM. After repair is completed TGFβ signalling is terminated, and ECM synthesis decreases to normal levels. However, in fibrotic diseases such as IPF, TGFβ signalling is not diminished leading to increase in synthesis and accumulation of ECM and fibrosis. TGFβ activity can be regulated by integrin-dependent activation and also by transcription factors such as nuclear receptor 4A1 (NR4A1) (Munger *et al.*, 1999; Palumbo-Zerr *et al.*, 2015). It has been shown that there is a NR4A1 feedback loop. In normal wound healing upregulation of TGFβ induces the expression of NR4A1 which in turn terminates TGFβ signalling (Palumbo-Zerr *et al.*, 2015). NR4A1 terminates TGFβ signalling through promoting the association of a repressor complex that binds to promoters of TGFβ target genes to inhibit their activation (Palumbo-Zerr *et al.*, 2015). In fibrotic disease the NR4A1 feedback loop is disrupted

by persistent TGFβ activity inactivating NR4A1 thus inhibiting its regulatory effects (Palumbo-Zerr *et al.*, 2015).

## TGFβ signalling in fibrosis

TGFβ is central to IPF development. TGFβ promotes epithelial cell apoptosis, epithelial cell migration, EMT, fibroblast proliferation, fibroblast transformation to myofibroblasts, and collagen synthesis (Goodwin and Jenkins, 2009). TGFβ has chemotactic properties causing the recruitment of macrophages and fibroblasts (Fernandez and Eickelberg, 2012). It also has proinflammatory effects by stimulating the expression of TNF-α, IL-1b, and IL-13 (Fernandez and Eickelberg, 2012). Both in pulmonary fibrotic tissue samples from animal models and patients with IPF, TGFβ levels are increased. Blocking TGFβ activity, through the TGFβ receptor blocks fibrosis of the lung in animal models (Bonniaud *et al.*, 2005). Overexpression of TGFβ is a widely-used animal model for pulmonary fibrosis studies (B Moore *et al.*, 2013).

TGFβ1 is the isoform which has been best characterised in IPF (Xaubet *et al.*, 2003). TGFβ1 is a pleiotropic cytokine that is ubiquitously expressed and produced by many different cells such as macrophages, neutrophils, epithelial cells, fibroblasts, and myofibroblasts. Its activity is regulated both temporally and spatially mainly through TGFβ1 being synthesised and released in an inactive form.

## Galectin-3

Galectins are a family of animal lectins that can bind to β-galactoside. There are fifteen galectins that have been found in mammals (Dumic, Dabelic and Flögel, 2006). Each galectin shares a core sequence in their carbohydrate recognition domain (CRD). However, each galectin exhibits structural differences in the CRD region. Galectins with one CRD are known as the prototypical galectins and exist as homodimers. These galectins include galectin 1, 2, 5, 7, 10, 11, 13, 14, 15. Galectins with two CRDs are the tandem galectins, and include galectins 4, 6, 8, 9, 12. Galectin-3 is the only galectin that exists as a chimera. It has one CRD which is linked to a proline, glycine, and tyrosine rich domain (the amino terminal domain) allowing galectin-3 to exist as a monomer or in a multimeric form up to a pentamer (in a concentration dependent manner) (Ahmad *et al.*, 2004). The ability of galectin-3 to exist as a monomer or an oligomer allows it to have many different biological functions (Nieminen *et al.*, 2007).

Galectin-3 is a 30kDa molecule. Human galectin-3 is coded by the LGALS3 gene found on q21-q22 on chromosome 14. Galectin-3 is a pleotropic molecule which is expressed within the nucleus, cytoplasm, and at the cell surfaces. Galectin-3 is also secreted by many different cell types including fibroblasts, epithelial cells, macrophages and monocytes (Sato and Hughes, 1994; Kasper and Hughes, 1996). Galectin-3 secretion can be stimulated by stress such as heat shock, injury, and irradiation (Sato and Hughes, 1994; Kasper and Hughes, 1996). Galectin-3 can bind

to cell surfaces, ECM proteins such as fibronectin and laminin, and receptors such as CD98 and CD45 (Dalton *et al.*, 2007; Xue *et al.*, 2013). It plays important roles in cell proliferation, adhesion, signalling, and apoptosis (Li, Li and Gao, 2014). The protein is also implicated in biological processes such as inflammation and angiogenesis (Li, Li and Gao, 2014). Galectin-3 has been shown to play roles in fibrotic diseases such as renal, liver, cardiac, and pulmonary (Kasper and Hughes, 1996; Henderson *et al.*, 2006, 2008; Nishi *et al.*, 2007; MacKinnon *et al.*, 2012).

It has been shown that galectin-3 can have both anti-apoptotic and pro-apoptotic effects. This depends on where galectin-3 is localised. Galectin-3 and Bcl-2 share significant sequence similarities where the CRD of galectin-3 contains the NWGR motif that is found within the BH1 domains of the Bcl-2 family. Accordingly, Intracellular galectin-3, acting through the Bcl-2 pathway can prevent cell death (Yang, Hsu and Liu, 1996). Whereas extracellular galectin-3, acting through the integrin β1 and CD7 induces apoptosis through the release of cytochrome c and the activation of caspase 3 (Fukumori *et al.*, 2003).

Galectin-3 has been shown to bind to components of the ECM such as laminin, fibronectin, collagen IV, and certain integrins (Sato and Hughes, 1992). Interactions with these components and by lattice formation and cross-linking allows galectin-3 to modulate cell adhesion. Because of the pro-apoptotic and pro-adhesive properties of galectin-3, a large number of studies have shown it to be involved in

cancer biology and metastasis (Liu, Patterson and Wang, 2002; Ochieng, Furtak and Lukyanov, 2002; Funasaka, Raz and Nangia-Makker, 2014; Ruvolo, 2016).

Galectin-3 is both highly expressed and secreted by macrophages (Henderson *et al.*, 2008). Hence it plays a major role in inflammatory responses. It has been shown that galectin-3 is involved in the activation and adhesion of neutrophils, attraction and migration of macrophages and the chemoattraction of monocytes. The expression of galectin-3 increases when monocytes differentiate into macrophages (Alison C MacKinnon *et al.*, 2008). CD98, a known receptor for galectin-3, is also expressed by macrophages. A study by Mackinnon *et al.* 2008, found that galectin-3 thorough acting at the CD98 receptor can lead to the activation of M2 macrophages. In addition, activation of the M2 macrophages was amplified through a galectin-3 feedback loop (Alison C MacKinnon *et al.*, 2008) where increased expression and secretion of galectin-3 by M2 macrophages further enhanced the activation of the alternative macrophages. In galectin-3 deficient mice it was demonstrated that there is an imbalance in Th1/Th2 cells (lower Th2 and increased Th1 cells).

A study by Partridge et al. (Partridge, 2004) delineated the effect of galectin-3 on receptor signalling. They show that via galectin-3 binding to the N-acetyllactosamine residues on epidermal growth factor receptor (EGFR) and both TGF-βRI and TGF-βRII, galectin-3 can prolong receptor signalling by delaying the removal of the receptor at the cell surface through endocytosis.

## Galectin-3 in fibrosis

A number of studies have provided evidence that galectin-3 is implicated in organ fibrosis. It has been shown to be involved in mechanisms involved in fibrotic disease development such as fibroblast proliferation, fibroblast transformation to myofibroblasts, and EMT (Henderson *et al.*, 2006, 2008; MacKinnon *et al.*, 2012). In quiescent fibroblasts the localisation of galectin-3 is mainly cytoplasmic, however during proliferation the localization of the protein changes to nuclear (Moutsatsos *et al.*, 1987). The different localisation of galectin-3 affects its role in cells. Galectin-3 was shown to be a mitogen for fibroblasts, where it was able to promote fibroblast proliferation. Galectin-3 is critical in TGFβ mediated myofibroblast activation, as galectin-3 siRNA prevented the activation of myofibroblasts (Henderson *et al.*, 2006).

In liver fibrosis its was shown by Henderson *et al*. that the expression of galectin-3 was upregulated in human liver fibrosis, and the expression of galectin-3 is temporally and spatially correlated with the fibrosis (Henderson *et al.*, 2006). They noted that in the liver fibrosis animal model, levels of galectin-3 increased both at the mRNA and protein level, which correlated to the increases in collagen deposition (Henderson *et al.*, 2006). In Galetin-3 -/- mice there was a significant decrease in collagen expression suggesting that galectin-3 regulates collagen deposition in liver fibrosis (Henderson *et al.*, 2006).

The expression of galectin-3 in an animal model of renal fibrosis was upregulated at both the protein and mRNA levels (Henderson *et al.*, 2008). Galectin-3 secreted by macrophages is critical to the activation of myofibroblasts (Alison C MacKinnon *et al.*, 2008; Henderson *et al.*, 2008). Galectin-3 -/- mice were protected against renal fibrosis with reduced collagen deposition and a reduction in activated myofibroblasts (Henderson *et al.*, 2008). This indicates that increased galectin-3 expression and secretion by macrophages consequently leads to the activation of collagen producing myofibroblasts and is a major mechanism in the development of renal fibrosis (Henderson *et al.*, 2008).

Galectin-3 has been established to be involved in TGFβ signalling in the development of IPF (MacKinnon *et al.*, 2012). Kasper *and collages* revealed that after irradiation induced lung injury in rats galectin-3 concentrations were significantly increased (Kasper and Hughes, 1996). The increase in galectin-3 following lung injury was attributed to increases in galectin-3 positive interstitial cells and alveolar macrophages. Type II alveolar epithelial cells serve as progenitors to type I epithelial cells. In normal lungs following lung injury, the type II epithelial cells migrate, proliferate, and re-epithelialize the damaged alveolar basement membrane. It was noted that the galectin-3 was predominantly expressed at the surfaces to the newly formed AT1 cells and to a lesser extent in AT2 cells suggesting that galectin-3 could be involved in normal repair by promoting re-epithelialisation (Kasper and Hughes, 1996). Persistent lung injury thus may disturb normal galectin-3 responses promoting fibrosis.

The earliest study investigating the role of galectin-3 in IPF had demonstrated that galectin-3 levels were increased in the bronchoalveolar lavage fluid (BALF) from patients with IPF compared with healthy patients (Nishi *et al.*, 2007). The expression of galectin-3 was greater in alveolar macrophages from patients with IPF compared with controls (Nishi *et al.*, 2007). They further revealed that galectin-3 induced migration and collagen synthesis by fibroblasts, and galectin-3 induced the production of TNF-α and IL-8 by macrophages (Nishi *et al.*, 2007).

In a study conducted by MacKinnon and colleagues (MacKinnon *et al.*, 2012), mice deficient in galectin-3 were protected from lung fibrosis in response to administration of TGFβ1 adenovirus and bleomycin. The extent of collagen deposition in the galectin-3 -/- mice compared to wild type mice treated with TGF-β1 adenovirus or bleomycin was reduced (MacKinnon *et al.*, 2012). In wild type mice treated with TGFβ1 adenovirus or bleomycin galectin-3 expression was increased in alveolar macrophages and bronchial epithelium and the BALF the expression of galectin-3 was temporally and spatially localised to fibrotic regions of the lungs (MacKinnon *et al.*, 2012). They showed that there were no difference in the expression of active TGFβ1 between wildtype mice and galectin-3 -/- mice treated with TGFβ1 adenovirus (MacKinnon *et al.*, 2012) indicating that galectin-3 plays a pivotal role in the development of lung fibrosis through an involvement in TGFβ1 signalling without affecting TGFβ expression.

Fibroblasts with galectin-3 knocked-out had reduced activation and transformation to myofibroblasts resulting in significantly reduced collagen production in response to TGFβ1 stimulation (MacKinnon *et al.*, 2012). Treatment with TGFβ1 in airway epithelial cells increased the release of galectin-3 (MacKinnon *et al.*, 2012). Galectin-3 -/- airway epithelial cells, in response to TGFβ1 treatment, showed reduced EMT compared to wild type suggesting that TGFβ1 induced EMT of epithelial cells is dependent on galectin-3 (MacKinnon *et al.*, 2012). A reduction in the cell surface galectin-3 led to a reduction in expression of TGFβRII (MacKinnon *et al.*, 2012). This is accredited to the reduction in TGF-βR retention and increase in receptor endocytosis, due to the loss of galectin-3 binding to the N-acetyllactosamine residues on TGFβRs (Partridge, 2004). Interestingly despite effecting TGFβRII retention there was no effect of galectin-3 on the canonical TGFβR signalling pathway (MacKinnon *et al.*, 2012). However, galectin-3 regulated EMT through a Smad-independent pathway, which involved Wnt signalling and the activation of β-catenin (MacKinnon *et al.*, 2012). Also knockdown of galectin-3 in the epithelial cells reduced TGFβ1 induced cell migration, highlighting galectin-3 is important in TGFβ induced responses. The novel galectin-3 inhibitor, TD139, reduced the amount of fibrosis in a bleomycin induced lung fibrosis model (MacKinnon *et al.*, 2012). Together these data suggest that galectin-3 maybe a potential therapeutic target for the treatment of IPF.

Figure 1.4 schematically demonstrates the hypothesised view of a galectin-3-TGFβ feedback loop. TGFβ stimulation of the TGFβ receptor leads to the

phosphorylation of Smad2. The activated smad2 translocates to the nucleus where it regulates the transcription of target genes including the galectin-3 gene, LGALS3. LGALS3 is upregulated leading to increase in galectin-3 at the cells surface and enhanced secreted galectin-3. Cell surface galectin-3 binds to the polylactosamine residues on TGFβ receptors, increasing cell surface receptor retention by preventing endocytosis, thus prolonging TGFβ receptor signalling. Galectin-3 secreted by the cell can further promote TGFβ signalling (MacKinnon *et al.*, 2012); directly by acting as a ligand and stimulating TGFβ receptor, and/or indirectly by activating latent TGFβ through/or indirectly through integrins.

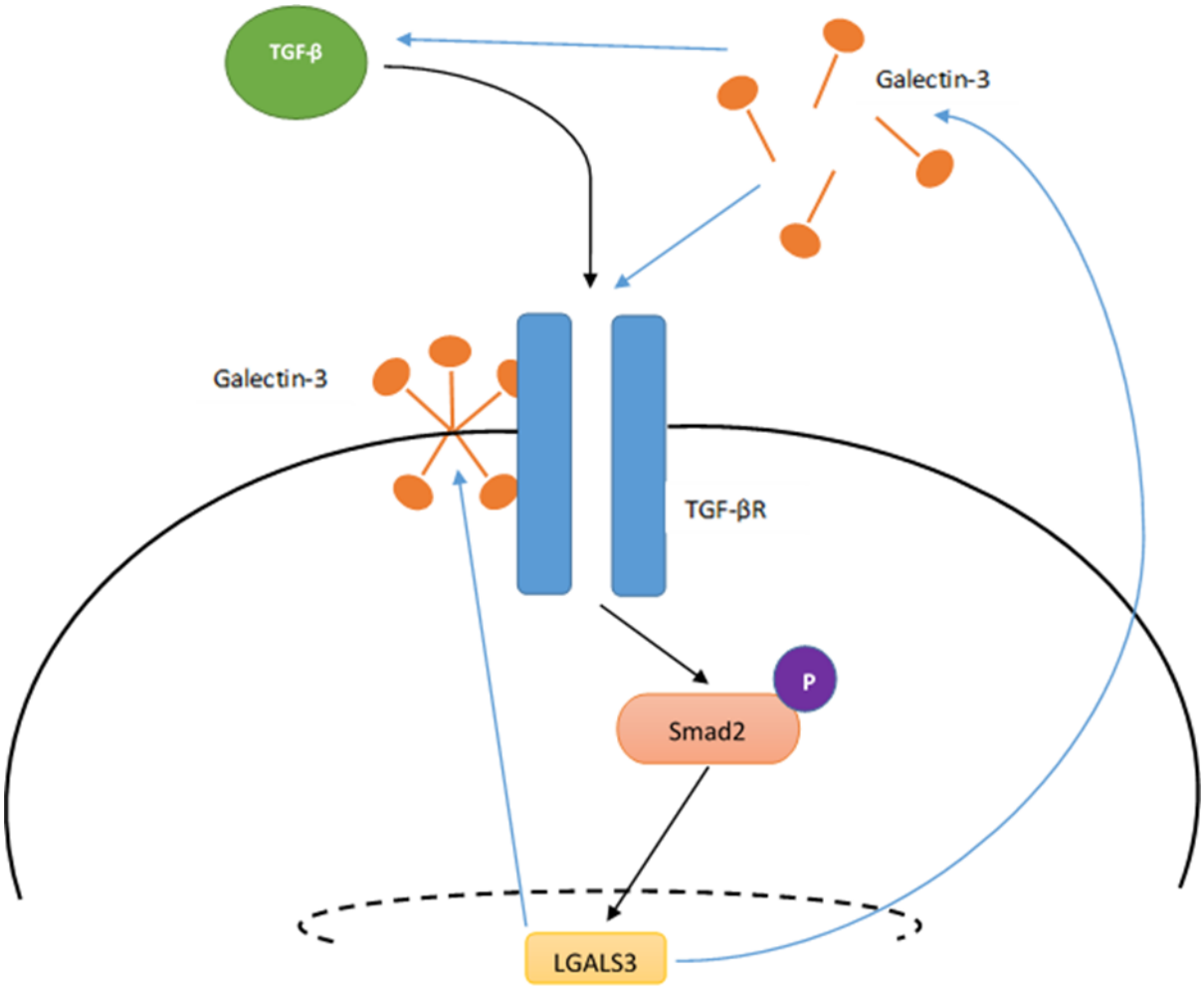



*Figure 1.4*

Proposed view of a Galectin-3-TGFβ feedback loop. TGFβ activates TGFβ receptor leading to the phosphorylation of Smad2. Ultimately leading to the upregulation of galectin-3 through increased expression of LGALS3 (MacKinnon *et al.*, 2012). The increase in levels of galectin-3 leads to the increase in galectin-3 at the cell surface. Cell surface galectin-3 prolongs TGFβ receptor signalling and activity by preventing its endocytosis (Partridge, 2004). TGFβ also increases the secretion of galectin-3 (MacKinnon *et al.*, 2012). Galectin-3 may be able to stimulate TGFβ signalling directly by activating the TGFβ receptors, and/or indirectly by activating latent TGFβ.

## Hypothesis

Galectin-3 is involved in TGFβ activity and signalling which is integral to IPF development.

# Aims

- To define the role of galectin-3 on TGFβ signalling in lung fibroblasts.
- To understand the role of galectin-3 on TGFβ signalling in lung epithelial cells.
- To dissect the mechanism through which galectin-3 promotes enhanced TGFβ activity in the lung.

# Chapter 2: Materials and Methods

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## Reagents and materials

### General reagents

Phosphate buffered saline (PBS) (Sigma Aldrich, P4417)

Tween-20 (Sigma Aldrich, P2287)

Methanol (Fisher Scientific, M4000/17)

Ethanol (Sigma Aldrich, E7023)

Bovine serum albumin (Sigma Aldrich, A7030)

Dimethyl sulphoxide (DMSO) (Sigma Aldrich, D5879)

### Cells and cell culture reagents

Mouse embryonic fibroblast cells expressing β6 integrin (MEFβ6) (transfected previously (Xu *et al.*, 2009) which stable cell lines were generated)

Transformed mink lung epithelial cells (TMLC) (gift from Dr Dan Rifkin, New York University, New York)

Human lung fibroblasts (HLFs) explanted from the lungs of healthy and IPF patients.

Immortalised bronchial epithelial cells (IHBECs) (gift from Professor Jerry Shay, University of Texas, USA).

Dubellcos Modified Eagles Medium (DMEM) (Sigma Aldrich, D5671)

Foetal calf serum (Thermo Fisher, 11573397)

Penicillin/streptomycin (Sigma Aldrich, P4458)

Trypsin-EDTA (Sigma Aldrich, T4049)

L-Glutamine (Sigma Aldrich, G7513)

## Cytokines and inhibitors

Recombinant human TGFβ (R&D, 240-B-010)

Recombinant human Galectin-3 (R&D, 1154-GA-050)

LPA (Sigma Aldrich, L7260)

Thrombin Receptor Agonist amide (SFLLR-NH2) (Cambridge Bioscience, ANA60680)

Scrambled TRAP Fragment (Cambridge Bioscience, ANA60197-1)

Alk5 inhibitor (Sigma Aldrich, S4317-5MG)

GB1211 inhibitor (Galecto Biotech, Denmark)

GB1107 inhibitor (Galecto Biotech, Denmark)

GB1211 inhibitor (Galecto Biotech, Denmark)

TD139 inhibitor (Galecto Biotech, Denmark)

Av inhibitor (gift from Professor David Griggs, Saint Louis University, USA)

Avβ5 inhibitor (ALULA) (gift from Professor Dean Sheppard, UCSF California, USA)

β1 inhibitor (gift from Professor Simon Macdonald, University of Nottingham)

## Immunohistochemistry reagents

Goat Serum (Sigma Aldrich, G9023)

Anti-galectin 3 antibody (ab76245)

Goat anti-rabbit IgG (Vector Laboratories, BA-1000)

Vectastain Elite ABC kit (Vector Laboratories, PK-6100)

DAB (Sigma Aldrich, D4418-50)

## Immunocytochemistry reagents

8 well chamber slides (Fisher Scientific, 354118)

Anti-galectin 3 antibody (ab76245)

ProLong Gold Antifade (Invitrogen)

## Western blot reagents

Pierce bicinchoninic acid (BCA) assay kit (Thermo Fisher, 10678484)

Acrylamide/Bis-acrylamide, 30% solution (Sigma, A3574)

Blotto non-fat dry milk (Santa Cruz, 2324)

GE Healthcare Rainbow™ Molecular Weight Markers (Thermo Fisher, RPN800E)

GE Healthcare Amersham™ Hyperfilm™ ECL (Fisher, 10752067)

Immun-Blot® PVDF Membrane (Bio-Rad, 1620177)

**Buffer 1**

| **Reagent** | **Amount** |
|---|---|
| Tris Base | 18.5g |
| 10% SDS | 4ml |
| dH20 | 50ml |

pH adjusted to 8.8 followed by addition of 100ml dH20.

**Buffer 2**

| Reagent | Amount |
|---|---|
| Tris Base | 6g |
| 10% SDS | 4ml |
| dH20 | 60ml |

pH adjusted to 6.8 then 100ml dH20 added.

**Resolving gel**

| Reagent | Amount |
|---|---|
| 30% Bis/acrylamide | 6.66ml |
| Buffer 1 | 5.2ml |
| dH20 | 7.92ml |
| 10% Ammonium Persulphate | 200µl |
| Tetramethylenediamine | 20µl |

**Stacking gel**

| Reagent | Amount |
|---|---|
| 30% Bis/acrylamide | 1.3ml |
| Buffer 2 | 2.5ml |
| dH20 | 6.1ml |
| 10% Ammonium persulphate | 50µl |
| Tetramethylenediamine | 10µl |

**Lamellae Buffer**

| Reagent | Amount |
|---|---|
| 0.5M Tris-HCl pH 6.8 | 5ml |
| 100% Glycerol | 4ml |
| 10% SDS | 4ml |
| Bromophenol Blue | 40mg |
| dH20 | 7ml |
| β-mercaptoethanol | 60µl/ml |

**10X Running Buffer**

| Reagent | Amount |
|---|---|
| Tris-base | 24.4g |
| Glycine | 144g |
| SDS | 10g |
| dH20 | 1000ml |

Diluted 1:10 with dH20 for 1X running buffer

**10X Transfer Buffer**

| **Reagent** | **Amount** |
|---|---|
| Tris-Base | 24.2g |
| Glycine | 144g |
| dH20 | 1000ml |

Diluted 1:10 with dH20 for 1X transfer buffer

**10X Tris Buffered Saline with Tween (TBST)**

| **Reagent** | **Amount** |
|---|---|
| Tris-HCL (pH 6.8) | 24.2g |
| NaCl | 87.6g |
| dH20 | 1000ml |
| Tween 20 | 10ml |

## Antibodies- primary and secondary

Phospho-Smad2 (Ser465/467) Rabbit mAb (Cell Signalling Technology, 138D4 #3108)

HRP-conjugated goat anti-rabbit secondary antibody (Dako, P0448)

Anti-Glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) (Abcam, ab181603)

smad2/3 Rabbit polyclonal Ab (Cell Signalling Technology, #3102)

## RNA extraction and qPCR reagents

NucleoSpin RNA II kit (Macharey Nagel, 740955)

Kapa SYBR Fast taq (Kapa Biosystems)

## Cyclic cell stretch reagents and equipment

6 well Bioflex plates (Dunnlabs, BF-300IU)

## ELISA reagents

Human galectin-3 quantikine ELISA kit (Bio-Techne, DGAL30)

## Co-Immunoprecipitation reagents

Universal Magnetic Co-IP Kit (Active Motif, 54002)

Anti-Integrin alpha V antibody [EPR16800] (Abcam, ab179475)

## Solid-phase binding assay reagents

Recombinant human LAP (R&D, 246-LP-025/CF)

Recombinant human LTBP1 (Abcam, ab114738)

## Co-culture reporter assay reagents

Luciferase (Promega, E1501)

# Equipment

Spectrophotometer (BMG Labtech, Ortenburg, Germany)

Nikon Eclipse 90i microscope

Nikon Ti confocal microscope

Mini-Protean Tetra cell electrophoresis sysetem (BioRad)

Transblot transfer apparatus (BioRad)

MXPro3000 thermocycler (Stratagene)

FacsCalibur (BD Bioscience)

FLOUstar omega multi-mode microplate reader (BMG Labtech, Ortenburg, Germany)

# General Methods

## MEFβ6 cell line culture

Mouse embryonic fibroblast cells with transfected β6 integrin (MEFβ6) were grown in Dubellcos Modified Eagles Medium (DMEM) containing 10% foetal calf serum, 4mmol/L L-Glutamine, and 5μg/mL Blastocidin (InVivoGen, San Diego, CA) in T150 flasks at $37^0$C in a humidified incubator with 5% $CO_2$. Blastocidin is a nucleoside antibiotic which inhibits protein synthesis in prokaryotic and eukaryotic cells. MEFβ6 were engineered to carry a resistance gene for Blastocidin, thus addition of the Blastocindin antibiotic to a MEFβ6 culture will ensure only MEFβ6 cells grow.

Passage of the cells was performed by aspiration of the medium and washing cells with phosphate buffered saline (PBS) three times. Trypsin-EDTA (5mls) was

added and cells were incubated at $37^0$C for 1min. Detachment of cells was visualised by brightfield microscopy. 10mls of DMEM was added to neutralise the trypsin and the trypsin-DMEM medium was centrifuged at 11,000 x g for 5mins. Supernatant was then removed, and cells resuspended in fresh DMEM. Cells were then counted, and the cell suspension split accordingly. Cells were approximately 80% confluent at the start of the experiment.

## TMLC cell line culture

Transformed mink lung epithelial cells (TMLC) which stably express firefly luciferase gene driven by the PAI-1 promoter were grown in DMEM containing 10% foetal calf serum, 4mmol/L L-Glutamine, and 250µg/ml G418 (Sigma-Aldrich) in T150 flasks at $37^0$C in a humidified incubator with 5% $CO_2$ in a humid environment. G418 is used as a selective agent for eukaryotic cells. As TMLCs are resistant to G418, addition of G418 ensures stable colonies of TMLCs are grown.

Passage of the cells was performed by as described previously.

## Healthy and IPF HLF cell line culture

IPF human lung fibroblasts were obtained from surplus lung parenchymal tissue from patients undergoing lung resections for the diagnosis of interstitial lung disease. These patients had a clinical diagnosis of IPF. Control fibroblasts (herein called healthy fibroblasts) were obtained from normal tissue distant from lung cancer

resections. The tissue collection was approved through the Nottingham University Hospital biobank and all patients had given written and informed consent. The tissue was dissected into small 3mm cubes and grown in T25 culture flasks. Fibroblasts were grown out in DMEM containing 10% foetal calf serum, 4mmol/L L-Glutamine, penicillin (200U/ml) and streptomycin (200µg/ml), and 5µg/ml amphotericin B. At passage 2, DMEM was changed and antibiotic concentrations were halved. Cells at passage 6 were used in the study.

Human lung fibroblasts (HLFs) from healthy and IPF patients were grown in DMEM containing 10% foetal calf serum, 4mmol/L L-Glutamine, penicillin (100U/ml) and streptomycin (100µg/ml) in T150 flasks at $37^0$C in a humidified incubator with 5% $CO_2$. For *in vitro* assays, experiments were replicated in three different healthy and three different IPF HLF cell lines.

Passage of the cells was performed by as described previously.

## IHBEC cell line culture

Immortalised bronchial epithelial cells (IHBECs) were cultured in keratinocyte serum free medium (Lonza) containing 25µg/ml bovine pituitary extract (Lonza), 0.2ng/ml epidermal growth factor (Lonza), 25µg/ml G418 (Sigma-Aldrich), 250ng/ml puromycin (Sigma-Aldrich) in T150 flasks at $37^0$C in a humidified incubator with 5% $CO_2$.

Passage of the cells was performed by as described previously.

## Cell counting

Cells were counted using a haemocytometer. Cell suspension (1µl) was added to the counting chamber and cells were counted using bright-field microscope. The counting chamber consists of nine large squares (in a 3x3 formation) with smaller square within (in a 4x4 formation). Cells in each large square were counted by the totalling the number of cells within each small square. The number of cells was calculated using the formula:

Cells/ml= average number of cell count in the small squares x $10^4$

## MTT assay

Mitochondrial enzymes in metabolically active cells cleave yellow tetrazolium dye MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) into insoluble formazan which is purple. The formazan is then solubilised by the addition of DMSO. The amount of purple formazan produced is directly proportional to the number of metabolically active cells present.

After experimental cell treatment, cell supernatant was removed, and cells washed with PBS. Cells were incubated with MTT in medium for 10mins at $37^0$C. The MTT solution was then discarded and plates were allowed to dry overnight at room

temperature. The cells containing the purple formazan were solubilised by the addition of DMSO. The absorbance was then read at 550nm using a spectrophotometer.

## Immunohistochemistry

### Working principle

Immunohistochemistry allows for the detection of proteins/antigens in tissue sections. Detection of proteins via this method allows for the ability to visualise the location and distribution of the protein of interest within the tissue or cells. The method involves a primary antibody binding to the interested antigen. This is followed by a horse radish peroxidase (HRP) conjugated secondary antibody binding to the bound primary antibody-antigen complex. The activity of the HRP allows for the visualisation and detection of the protein of interest.

Prior to the immunostaining, the tissue is collected and preserved in formalin and embedded in paraffin wax. Formalin helps preserve the structural integrity and tissue architecture. Sections of tissue are cut using a cryostat.

Following the addition of the primary and secondary antibodies, DAB (3,3'-Diaminobenzidine) is added to visualise the target protein. DAB is oxidised by hydrogen peroxide from a reaction catalysed by HRP found on the secondary

antibody. The oxidised DAB forms a brown precipitate at the location of the HRP which can then be visualised via light microscopy.

## Immunohistochemistry protocol

Lung tissue collection was approved and obtained through the Nottingham University Hospital biobank and all patients had given written and informed consent. Table 2.1 summaries lung tissue source and clinical diagnosis of the lung tissues used for immuno-staining. IPF lung tissue was used for immunohistochemistry from patients that had a pathological diagnosis of usual interstitial pneumonia and a clinical diagnosis of IPF. Normal lung tissue from patients with no clinical diagnosis of lung cancer of interstitial pneumonia was used as control (herein called healthy lung tissue).

| **Sample I.D** | **Disease type** |
|---|---|
| RS10005 | Healthy |
| 12/002773/T00/FP10 | Healthy |
| 12/001861/T00/FP01 | Healthy |
| AH20061401 | IPF |
| GM300114A | IPF |
| HF15113A | IPF |
| CP400878T/16 | IPF |

*Table 2. 1*

Histological sections of lung tissue were cut at 5µm. Paraffin-embedded sections of mouse lung tissue and human lung tissue were immunostained with anti-galectin-3 antibody. Lung sections were deparaffinised in xylene for 1min followed by rehydration in graded alcohol (3mins in 100% ethanol, 70% ethanol and $H_2O$). As the fixation of tissue can alter the protein biochemistry of the protein of interest through methylene bridges which can mask antigen sites an antigen retrieval step needs to be performed. This constitutes of sections were incubated in 10mM sodium citrate buffer for 10mins. Following this endogenous peroxidase was blocked using 3% $H_2O_2$ for 30mins. After washing in PBST for 5mins three times, 5% goat serum was added to the lung sections to prevent non-specific antibody binding. Sections were incubated overnight with anti-galectin 3 antibody (ab76245) at a dilution of 1:6000 . Sections were then incubated with the secondary antibody- biotinylated-goat anti-rabbit IgG (Vector Laboratories, BA-1000) at a dilution of 1:200 for 1 hour followed by the Vectastain Elite ABC kit (Vector Laboratories, PK-6100) for 30mins. Peroxidase activity was visualised by the application of DAB. Tissue sections were counterstained with haematoxylin, dehydrated by placing the sections in 70% then 100% ethanol for 2mins, and mounted ready for microscope analysis. Images were captured by Nikon Eclipse 90i microscope and NIS Elements v3.2 software.

# Immunocytochemistry

## Working principle

Immunocytochemistry is a protein detection method where the protein binding to the antigen is visualised using a fluorophore. It offers a semi-quantitative way of visualising the abundance and localisation of the antigen of interest.

## Immunocytochemistry protocol

Cells were seeded onto a 4-chamber slide and incubated at 37$^{o}$C at 5% $CO_2$. Following treatment of the cells, medium was aspirated, and cells were fixed via the addition of ice-cold methanol. The cells were store at -20$^{o}$C until analysis. Methanol was then removed, and cells were washed three times with PBS. Non-specific antibody binding was blocked by addition of with 1% BSA for 1 hour room temperature. The cells were then washed, and primary antibody was added which was diluted at 1:1000 in 0.01% BSA and incubated overnight at 4$^{o}$C. Primary antibody was then removed, and fluorescent dye labelled secondary antibody along with DAPI was added and incubated for one hour at room temperature. The secondary antibody was then removed, and cells washed with PBS. The cells were then mounted with ProLong Gold Antifade (Invitrogen) and analysed using a confocal microscope. Images were captured via Nikon Ti confocal microscope with NIS Elements Software v3.2.

# Western blot

## Working principle

Western blotting produces quantitative data of proteins of interest from cell or tissue lysates. The technique relies on proteins being separated into a polyacrylamide gel according to their molecular weight. The separated proteins are then transferred onto either a nitrocellulose or PVDF membrane. After blocking the membrane to reduce non-specific binding site, the membrane is incubated with a specific primary antibody and an enzyme labelled secondary antibody. The addition of a specific chemiluminescent substrate generates a light signal. The intensity of the light signal is dependent on the quantity of bound enzyme labelled secondary which in turn is dependent on the amount of bound primary antibody to the protein of interest.

## Western blot protocol

Following treatment, cells were washed once in ice cold PBS and 150µl of lysis buffer containing protease inhibitors, phosphatase inhibitors, phenylmethanesulfonylfluoride flouride (PMSF) and PBS. PMSF is a serine protease inhibitor and is added to the lysis buffer to prevent protein degradation. The lysed cells in the 6-well plated on ice to prevent protein degradation were then stored at -80$^{o}$C to await analysis.

Cells were then scraped and transferred to microcentrifuge tubes. The samples were then centrifuged at 14,000 x g for 10mins at 4$^{o}$C. The supernatants were transferred to microcentrifuge tubes and the pellets were discarded. The protein concentration was then determined using the Pierce bicinchoninic acid (BCA) assay. The purple product formed by the chelation of bicinchoninic acid by $Cu^{+1}$ is measured by colometry. 5µl of sample or BCA standard (0-2000µg/ml) was added to individual wells in a 96-well plate. 95µl BCA reagent, supplied in the BCA kit, was then added to each well followed by incubation at room temperature for 30mins. The plate was then read using a spectrophotometer at 560nm. The protein concentration was calculated by comparing the absorbance of the samples to a linear standard curve of absorbance of the BCA standards.

Laemmli buffer sample buffer containing 2.5% β-mercaptoethanol was then added to 15-30µg of sample. The samples were then denatured on a heat block at 100$^{o}$C for 5mins. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE). Gels were prepared (see western blot reagents) by the addition of resolving gel solution and stacking gel solution to the gel casting system. 15-30µg of protein was loaded per lane in a 10% SDS-polyacrylamide gel. A molecular weight marker was added to one lane at each side of the gel to assess the molecular weights of inspected proteins. The proteins were separated in the 10% gel in running buffer at 150V.

After the electrophoresis stage of the western blot, protein was transferred from the gel to a polyvinylidene difluoride membrane (PVDF) using transfer buffer at 110V for 1 hour. After the transfer stage, the PVDF membranes were blocked with 5% milk (5% non-fat milk in Tris Buffered Saline plus 0.1% Tween 20 (TBST)) for one hour at room temperature. The membranes were then incubated overnight at 4$^{o}$C with primary antibody. Following overnight primary antibody incubation membranes were washed with TBST and then incubated with Horse Radish Peroxidase (HRP)-conjugated secondary antibody for one hour at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL.

To investigate phosphorylated Smad2, membranes were then incubated overnight at 4$^{o}$C with anti-pSmad2 primary antibody (Phospho-Smad2 (Ser465/467) (138D4) Rabbit mAb #3108, Cell Signalling Technology) at 1:1000 dilution in 5% skimmed milk TBST. Following overnight primary antibody incubation membranes were washed with TBST and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (P0448, Dako) (1:3000 dilution in blocking buffer) for 1 hour at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL.

Following visualisation of Psmad2, membranes were probed further for anti-Glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) (1:10000 dilution in blocking buffer) for 1hour room temperature. Membranes were washed in TBST and then be incubated with HRP-conjugated goat anti-rabbit secondary antibody (P0448,

Dako) (1:3000 dilution in blocking buffer) for 1 hour at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL. GAPDH was used as the loading control.

Following visualisation of GAPDH, membranes were stripped using stripping buffer for 5 minutes. Membranes were washed in TBST and blocked with 5% milk (5% non-fat milk in TBS plus 0.1% Tween 20) for 1 hour at room temperature. The membranes were then incubated overnight at 4$^{o}$C with anti-smad2/3 primary antibody (smad2/3 Rabbit polyclonal Ab #3102, Cell Signalling Technology) at 1:1000 dilution in blocking buffer. Following overnight primary antibody incubation membranes were washed with TBST and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (P0448, Dako) (1:3000 dilution in blocking buffer) for 1 hour at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL.

The western blot bands were quantified using ImageJ software. Results are expressed as a band density ratio of pSmad2 to Smad2, which indicates the relative expression of pSmad2.

# qPCR

## Working principle

During PCR, sequences of DNA are amplified through the recurrent cycles of DNA polymerase catalysed reactions. Therefore, it allows for the DNA replication for the target DNA sequence to be selectively amplified. This leads to the synthesis of complementary DNA (cDNA). The RT-PCR product can then be quantified by using oligonucleotide primers which emit fluorescent signals upon cleavage or hybridisation. The threshold cycle (Ct) describes the number of cycles for the fluorescent signal to exceed the threshold level. It is inversely proportional to the amount of target nucleic acid in the sample. Thus, the higher the Ct the lower the amount of target nucleic acid. The data is compared to a standard curve to allow for quantification relative to a reference gene which is not affected by treatment. $^{\Delta\Delta}$Ct describes the difference between the Ct of the target nucleic acid and the Ct of the reference gene.

## qPCR protocol

The changes in gene expression at mRNA level of the galectin-3 gene (LGALS3) in response to 2ng/ml TGFβ after 24 hours was investigated by qPCR using MXPro3000 thermocycler (Stratagene) and Kapa SYBR Fast taq (Kapa Biosystems).

After cellular stimulation, total cell RNA was extracted using NucleoSpin RNA II kit (Macharey Nagel) and reverse transcribed into complimentary DNA (cDNA)

using Moloney murine leukemia virus transcriptase. Cells were lysed via the addition on 350µl RA1 lysis buffer (buffer from kit) and 3.5µl β-mercaptoethanol and transferred to microcentrifuge tubes. 350µl 70% ethanol was then added to the sample and then transferred to the NucleoSpin RNA Column tubes. The samples were then centrifuged for 30s at 11,000 x g. 350µl membrane desalting buffer was then added, and samples then centrifuged for 1 min at 11,000 x g. 95µl DNase reaction was then added to the sample and incubated at room temperature for 15mins. The samples were then washed in RAW2 buffer (from kit) and then centrifuged at 11,000 x g, RA3 buffer (from kit) and again centrifuged at 11,000 x g. The RNA was then eluted in 40µl of RNase-free $H_2O$. The concentration of the RNA was then determined using a NanoDrop spectrophotometer.

For the reverse transcription step, eluted RNA was made up to 10µl in RNase-free $H_2O$ and then mixed with reverse transcription components containing: 5x RT buffer (5µl), Oligo DT (3µl), 10mM dNTP (5µl), $H_20$ (0.7µl), RNAsin (0.65µl) and MMLRT (0.65µl). The RNA-RT mixture was added to a thermal cycler to synthesise the cDNA. The obtained cDNA was diluted 1:5 by adding 80µl $H_20$.

The cDNA was then quantified via qPCR. Human β2 mircoglobulin (β2M) was used as the house keeping gene 5'-AATCCAAATGCGGCATCT-3' (sense) and 5'-GAGTATGCCTGCCGTGTG-3' (anti-sense). Expression of human LGALS3 5'-AGATTTCCAAAGAGGGAATG-3' (sense) and 5'-AAGTGCAAACAATGACTCTC-3' (anti-sense) was measured. Programme was set to denaturing at 95°C for 30 seconds,

followed by 95°C for 5 seconds for 40 cycles, and 60°C for 30 seconds, and 72°C for 15 seconds. Data are expressed as relative expression using the $^{\Delta\Delta}$Ct equation.

## Cyclic cell stretch

Healthy and IPF HLFs were plated onto 6 well Bioflex plates. These plates contain a flexible silicone membrane which the cells adhere to. Cyclical stretch is created by the Flexcell tension system (FX5000T) whereby a vacuum is created in the gasket on which the Bioflex plates are seated therefore deforming the flexible membrane on which the cells are adhered to by pulling the membrane downwards and creating equiaxial stretch. Cells are approximately 80% confluent at the start of the experiment. Once 80% confluent, cells are serum starved overnight. After serum starvation, media is removed, and treatments added: 50µM Alk5 inhibitor and 1µM TD139 to the relevant wells. The 6 well plates are then installed onto the gasket of the Flexcell tension system. To create a normal physiological environment, a stretch frequency of 20 cycles per minute (0.33Hz) was applied and elongation strain of 15%, which is equivalent to parameters seen in people.

## ELISA

### Working principle

Enzyme linked immunosorbent assay (ELISA) is a technique used to quantify solid-phase bound proteins or proteins in solution using antibodies. In a sandwich ELISA, a capture antibody to the target protein is immobilised on the surface of the

plate. The plate is incubated with the sample containing the protein of interest followed by the target protein specific antibody which is labelled with an enzyme. The activity of the plate bound enzyme is measured using substrate solution and the optical density is read.

### ELISA protocol

Sandwich ELISA was used to detect human galectin-3 released into the media from HLFs. Following cell stimulation, the cellular supernatant was collected and stored at -80$^{o}$C prior to analysis via ELISA. 100µl of assay diluent was added to each well and 50µl of sample or standard was added to the wells and incubated for two hours at room temperature. Wells were then aspirated and washed four times using the wash buffer supplied in the kit. 200µl of human galectin-3 conjugated antibody was then added to each well and incubated at room temperature for two hours. Wells were then aspirated and washed four times using the wash buffer. 200µl of substrate solution was then added to the wells and incubated for 30mins at room temperature and protected from light. 50µl of stop solution (0.16M sulfuric acid) was then added to each well and the optical density was measured at 450nm and corrected at 570nm.

## Flowcytometry

Cells were grown in T150 flasks until approximately 80% confluent. Media was aspirated from cells and cells were trypsinised and counted then adjusted to 100,000

cells in 100µl PBS and sodium azide. Non-specific binding was blocked in 5% goat serum for 30mins. CD98 primary antibody (abcam, ab2528) at 5µg/ml was added to cells and incubated for 30mins at 4$^{o}$C. 1ml PBS and sodium azide was then added to wash the cells, centrifuged and supernatants removed. The cellular pellet was resuspended in 200µl PBS plus sodium azide. Secondary goat anti-mouse antibody conjugated with phycoerythrin (New England Bio Labs) was added (1:200 dilution) and incubated for 30mins at 4$^{o}$C. Cells were again washed with PBS, centrifuged and responded in PBS and sodium azide. The fluorescence was then quantified via mean fluorescence intensity on 100,000 cells with FacsCalibur with FlowJo version 10.1 software.

## Co-immunoprecipitation

### Working principle

Co-immunoprecipitation is an experimental technique which identifies protein-protein interaction. The technique involves the use of antibodies which capture proteins bound to target proteins. These protein complexes can be analysed to detect protein-protein binding interactions. The antibody against the protein of interest forms an immune complex with the target in the sample. The immune complex is precipitated and then analysed via western blot.

### Co-immunoprecipitation protocol

Co-immunoprecipitation was prepared according to the Universal Magnetic Co-IP Kit (Active Motif) protocol and reagents. Once HLFs were treated, lysed and concentration determined via BCA assay, 100µg of protein in addition of 500µl wash buffer and primary antibody (1mg/ml IgG, or 1:30 dilution galectin-3 primary antibody, αv integrin primary antibody 1:40 dilution) was incubated on a rotator for 2 hours at 4°C. The samples were centrifuged at 11,000 x g for 30 s and supernatants were collected. 25µl protein G magnetic beads were added to each sample and samples were incubated for one hour at 4°C on a rotator. Samples were then centrifuged for 30 seconds at 11,000 x g and the supernatant collected. The samples were then placed onto a magnetic stand and supernatant removed and the pellet resuspended in 20µl 4x LDS sample buffer containing 2.5% β-mercaptoethanol.

## Solid-phase binding assay

### Working Principle

Solid-phase binding assay is a technique used to investigate protein-protein interactions. The protein of interest in immobilised on the surface of the plate. The plate is incubated with the second protein of interest followed by the target protein specific antibody which is labelled with an enzyme. The activity of the plate bound enzyme is measured using substrate solution and the optical density is read.

### Solid-Phase binding assay protocol

High binding 96-well plates were coated with 2µg/ml recombinant TGFβ, or recombinant LAP, or recombinant LTBP1, or collagen, or PBS. The plates were incubated overnight to allow binding of the recombinant protein to the plate. Plates were then washed with PBS and blocked with 1% Bovine Serum Albumin for one hour. Plates were washed again with PBS and increasing concentrations of recombinant human galectin-3 (0, 0.01, 0.1, 1, 10µg/ml) was added the individual wells and incubated at room temperature for 4 hours. Wells were then washed with PBS and 100µl conjugated galetin-3 antibody was added to each well and incubated for 2 hours at room temperature. Wells were again washed and 100µl substrate solution was added and incubated for 30mins. 50µl stop solution was added and optical density was read using a spectrophotometer at 450nm with correction at 570nm.

## Co-culture reporter assay

### Working principle

Previous data from the lab had shown that αvβ6 binding to the latent TGFβ complex leads to TGFβ activation. The αvβ6 integrin is expressed by epithelial cells, thus MEFs were transfected to stably express the β6 integrin. TMLCs used in the experiment were transfected to express the luciferase gene driven by the PAI-1 promoter. PAI-1 is a TGFβ target gene thus, when grown in co-culture, the β6 integrin on MEFβ6 cells bind to the latent TGFβ complex leading to TGFβ activation.

TGFβ acting at the TMLCs leads to the activation of PAI-1 giving rise to luciferase synthesis. Consequently, the luciferase activity is measured as light emitted and the higher the luminescence the greater the TGFβ activity.

### Co-culture reporter assay

MEFβ6 cells were seeded in a 96 well plate at a density of $5x10^4$ cells/ml. TMLCs were seeded with the MEFβ6 in direct co-culture, and also seeded alone at a density of $5x10^4$ cells/ml to measure the total active TGFβ. The galectin-3 Inhibitors was added at a concentration dependent manner (1nM-1000nM). Recombinant human TGFβ1 was added to relevant wells in a concentration dependent manner (62.5pg/ml-5000pg/ml). TGFβ1 treatments was used as a standard curve. After 16h incubation at 37°C, cells were washed in cold PBS and lysed with 50µl of reporter lysis buffer (Promega). The lysates were then transferred to a luminometry plate, and 100µl of luciferase reagent was added. Subsequently, the luciferase activity was immediately measured using FLOUstar omega multi-mode microplate reader (BMG Labtech, Ortenburg, Germany). All experiments were performed in triplicate and were repeated at least three times.

## Statistical analysis

Data is presented as mean ± SEM and was analysed via non-parametric methods. Unless specified data was analysed using the Kruskal-Wallis test followed by Dunn's posthoc test to test between the treatment groups. Statistical analysis was

performed using GraphPad Prism version 7. P values less than 0.05 were considered statically significant. Experiments were replicated for a minimum of at least three times.

# Chapter 3: Galectin-3 expression in IPF

# Chapter 3 – Galectin-3 expression in IPF

## Introduction

IPF is a chronic, irreversible and progressive disease of the lungs. Prognosis for the disease is poor, with a median survival from the time of diagnosis is approximately 2-3 years (Ley, Collard and King, 2011). There currently is no curative treatment for IPF.

The pathogenesis of the disease is poorly understood. However, the current hypothesis of IPF development and progression involves repeated injury to the lung epithelium in a genetically susceptible individual. This is followed by dysregulated wound repair responses involving fibroblast migration to the site of injury, fibroblast activation, and fibroblast transformation into myofibroblasts. Additionally, epithelial cell injury leads to the loss of integrity to the alveolar epithelial membrane resulting in increased infiltration of inflammatory cells such as macrophages. Myofibroblasts continuously secrete ECM proteins such as collagen which leading to increased deposition. The ineffective ECM removal leads to ECM accumulation and increased airway stiffness. This leads to fibrosis and the progressive nature of the disease.

Galectin-3 is a member of the β-galactoside binding protein class of family called galectins. Out of the fifteen galectins within the galectin family, because of its unique N-terminal domain, galectin-3 is able to exist as a monomer, homodimer or

cluster with other galectin-3 monomers to form a pentamer structure. This unique property of galectin-3 allows it to have an enhanced binding and clustering ability.

Galectin-3 is ubiquitously expressed, where it is found within in nucleus of some cells, cytoplasm, at the cell surface, and also in the extracellular spaces as it is released by some cell types. It has been shown to be expressed by different cell types such as macrophages, epithelial cells, and fibroblasts (Sato and Hughes, 1994; Kasper and Hughes, 1996). Galectin-3 has numerous biological roles. It plays important roles in cell proliferation, adhesion, signalling, and apoptosis (Li, Li and Gao, 2014). It can bind to ECM proteins such as laminin and fibronectin, and receptors such as CD98 and TGFβ receptor (Partridge, 2004; Dalton *et al.*, 2007; Lau *et al.*, 2007; MacKinnon *et al.*, 2012).

In disease, numerous studies have highlighted the importance galectin-3 plays in the development of organ fibrosis (Kasper and Hughes, 1996; Henderson *et al.*, 2006, 2008; Nishi *et al.*, 2007; MacKinnon *et al.*, 2012). The expression of galectin-3 has been shown to be increased in fibrosis (Henderson *et al.*, 2006, 2008; MacKinnon *et al.*, 2012). Nishi and colleagues had shown that in patients with IPF galectin-3 concentrations were increased in the BALF compared to healthy individuals (Nishi *et al.*, 2007). Mice deficient in galectin-3 were protected from experimentally induced lung fibrosis (MacKinnon *et al.*, 2012). They showed that the extent of collagen deposition in the galectin-3 -/- mice treated with TGFβ1

adenovirus or bleomycin were dramatically reduced compared to the wild type mice (MacKinnon *et al.*, 2012).

Growth factors such as TGFβ have been shown to play a central role in pulmonary fibrosis. TGFβ1 is the isoform best characterised in IPF (herein called TGFβ for rest of the thesis) (Xaubet *et al.*, 2003). TGFβ has been shown to play roles in fibrotic pathways involved in epithelial cell apoptosis, ineffective epithelial cell migration, epithelial cell to mesenchymal cell transition (EMT), fibroblast activation, fibroblast transformation to myofibroblast, synthesis of collagen by myofibroblasts (Hagimoto *et al.*, 2002; Kim *et al.*, 2006; Scotton and Chambers, 2007; Yu *et al.*, 2008). TGFβ is a pleiotropic cytokine which is ubiquitously expressed and synthesised by various cells such as macrophages, neutrophils, epithelial cells, fibroblasts, and myofibroblasts.

Interestingly, wild type mice where TGFβ was overexpressed had an increased galectin-3 expression in macrophages, epithelium, and the BALF (MacKinnon *et al.*, 2012). Furthermore, the group had shown that there was no difference between the expression of active TGFβ in wildtype mice and galectin-3 -/- mice, demonstrating that galectin-3 regulates TGFβ activity.

## Aims and Objectives

The role of galectin-3 in the development and progression of pulmonary fibrosis is currently unknown. The understanding of which cell types produce, express, and release galectin-3 in IPF disease is also limited. It is hypothesised that in in IPF and in animal models of IPF that galectin-3 expression is increased. Thus, the aims of this chapter are to:

1. To investigate the cellular expression of galectin-3 in IPF disease.
2. To investigate the cellular expression of galectin-3 in *in vivo* models of pulmonary fibrosis.
3. To investigate the specific cellular expression and release of galectin-3 in *in vitro* models of pulmonary fibrosis.

# Methods

## Immunohistochemistry

Histological sections of lung tissue were cut at 5µm. Paraffin-embedded sections of mouse lung tissue and human lung tissue were immunostained with anti-galectin-3 antibody. Human lung tissue from patients with IPF and healthy controls (as described in chapter 2) were immuno-stained for galectin-3. Mouse lung tissue was taken from mice treated with bleomycin or saline control for 28 days (samples obtained from previous study and described in study by (Habgood *et al.*, 2016).

Lung sections were deparaffinised in xylene for 1min followed by rehydration in graded alcohol (3mins in 100% ethanol, 70% ethanol and $H_2O$). Sections were incubated in 10mM sodium citrate buffer for 10mins. Following this endogenous peroxidase was blocked using 3% $H_2O_2$ for 30mins. After washing in PBST for 5mins three times, 5% goat serum was added to the lung sections to prevent non-specific antibody binding. Sections were incubated overnight with anti-galectin 3 antibody (ab76245) at a dilution of 1:6000. Sections were then incubated with the secondary antibody- biotinylated-goat anti-rabbit IgG (Vector Laboratories, BA-1000) at a dilution of 1:200 for 1 hour followed by the Vectastain Elite ABC kit (Vector Laboratories, PK-6100) for 30mins. Peroxidase activity was visualised by the application of DAB. Tissue sections were counterstained with haematoxylin, dehydrated by placing the sections in 70% then 100% ethanol for 2mins, and

mounted ready for microscope analysis. Images were captured by Nikon Eclipse 90i microscope and NIS Elements v3.2 software.

## qPCR

The changes in gene expression at mRNA level of the galectin-3 gene (LGALS3) in response to 2ng/ml TGFβ after 24 hours was investigated by qPCR using MXPro3000 thermocycler (Stratagene) and Kapa SYBR Fast taq (Kapa Biosystems). Normal and IPF human lung fibroblasts (HLFs) and immortalised human bronchial epithelial cells (IHBECs) were treated with 2ng/ml TGFβ for 24 hours. After cellular stimulation, total cell RNA was extracted using NucleoSpin RNA II kit (Macharey Nagel) and reverse transcribed into complimentary DNA (cDNA) using Moloney murine leukemia virus transcriptase. The cDNA was then quantified via QPCR. Human β2 mircoglobulin (β2M) was used as the house keeping gene 5'-AATCCAAATGCGGCATCT-3' (sense) and 5'-GAGTATGCCTGCCGTGTG-3' (anti-sense). Expression of human LGALS3 5'-AGATTTCCAAAGAGGGAATG-3' (sense) and 5'-AAGTGCAAACAATGACTCTC-3' (anti-sense) was measured. Programme was set to denaturing at 95$^{o}$C for 30 seconds, followed by 95$^{o}$C for 5 seconds for 40 cycles, and 60$^{o}$C for 30 seconds, and 72$^{o}$C for 15 seconds. Data is expressed as relative expression using the ΔΔCt equation.

## ELISA

Sandwich ELISA Human galectin-3 quantikine ELISA kit (Bio-Techne, DGAL30) was used to detect human galectin-3 released into the media from HLFs. Normal and IPF HLFs and IHBECs were treated with 2ng/ml TGFβ for 24 hours. Following cell stimulation, the cellular supernatant was collected and stored at -80$^{o}$C prior to analysis via ELISA. 100µl of assay diluent was added to each well and 50µl of sample or standard was added to the wells and incubated for two hours at room temperature. Wells were then aspirated and washed four times using the wash buffer supplied in the kit. 200µl of human galectin-3 conjugated antibody was then added to each well and incubated at room temperature for two hours. Wells were then aspirated and washed four times using the wash buffer. 200µl of substrate solution was then added to the wells and incubated for 30mins at room temperature and protected from light. 50µl of stop solution (0.16M sulfuric acid) was then added to each well and the optical density was measured at 450nm and corrected at 570nm.

## Immunocytochemistry

Cells were seeded onto a 4-chamber slide and incubated at 37$^{o}$C at 5% $CO_2$. The cells were treated with 2ng/ml TGFβ for 2, 4 and 48 hours. Following treatment of the cells, medium was aspirated, and cells were fixed via the addition of ice-cold methanol. The cells were store at -20$^{o}$C until analysis. Methanol was then removed,

and cells were washed three times with PBS. Non-specific antibody binding was blocked by addition of with 1% BSA for 1 hour room temperature. The cells were then washed, and primary antibody was added which was diluted at 1:1000 in 0.01% BSA and incubated overnight at 4$^{o}$C. Primary antibody was then removed, and fluorescent dye labelled secondary antibody along with DAPI was added and incubated for one hour at room temperature. The secondary antibody was then removed, and cells washed with PBS. The cells were then mounted with ProLong Gold Antifade (Invitrogen) and analysed using a confocal microscope. Images were captured via Nikon Ti confocal microscope with NIS Elements Software v3.2.

# Results

## Galectin-3 expression in disease and bleomycin-induced injury

The cellular expression of galectin-3 in healthy and IPF human lung sections were immuno-stained for galectin-3. Galectin-3 expression was increased in the lungs of IPF compared with healthy patients (figure 3.1). The Increase in expression of galectin-3 is seen in cells that morphologically appear to be fibroblasts (indicated by the red arrows in figure 3.1C and 3.1D) and macrophages (indicated by the black arrow in figure 3.1D).

Additionally, the increased galectin-3 expression is noted within areas of fibrosis in the lung sections, where non-fibrotic areas were devoid of galectin-3 staining (figure 3.2A and 3.2B vs figure 3.2C, 3.2D, 3.2E and 23.F). However, areas of normal lung tissue did have positively stained macrophages present (indicated by the black arrows in figure 3.2A and 3.2B). Specifically, the increases in galectin-3 expression in the human lung sections were seen in epithelial cells (indicated by the blue arrows in figure 3.2C and 3.2D), macrophages (indicated by black arrows in figure 3.2) and fibroblasts (indicated by red arrows in figure 3.2). figure 3.2E and 3.2F is an example of fibroblastic foci seen in the lung of IPF (indicated by the yellow star). Galectin-3 expression is heavily expressed within these areas within the fibroblasts (indicated by red arrows).

The bleomycin mouse model is a widely used *in vivo* model for lung for lung fibrosis research. Figure 3.3 shows galectin-3 immunohistochemistry staining of mouse lung section from mice treated with 28 days of bleomycin or saline control. Galectin-3 expression was not observed cells of saline treated animals (figure 3.3A). However, the expression of galectin-3 was increased in bleomycin treated mice (figure 3.3B). The increased galectin-3 expression was seen in macrophages (indicated by black arrows in figures 3.3C and 3.3D) and alveolar epithelial cells (indicated by blue arrows in figures 3.3C and 3.3D). However, unlike within human IPF (see figure 3.2), the expression of galectin-3 in fibroblasts of bleomycin treated mice was not observed.

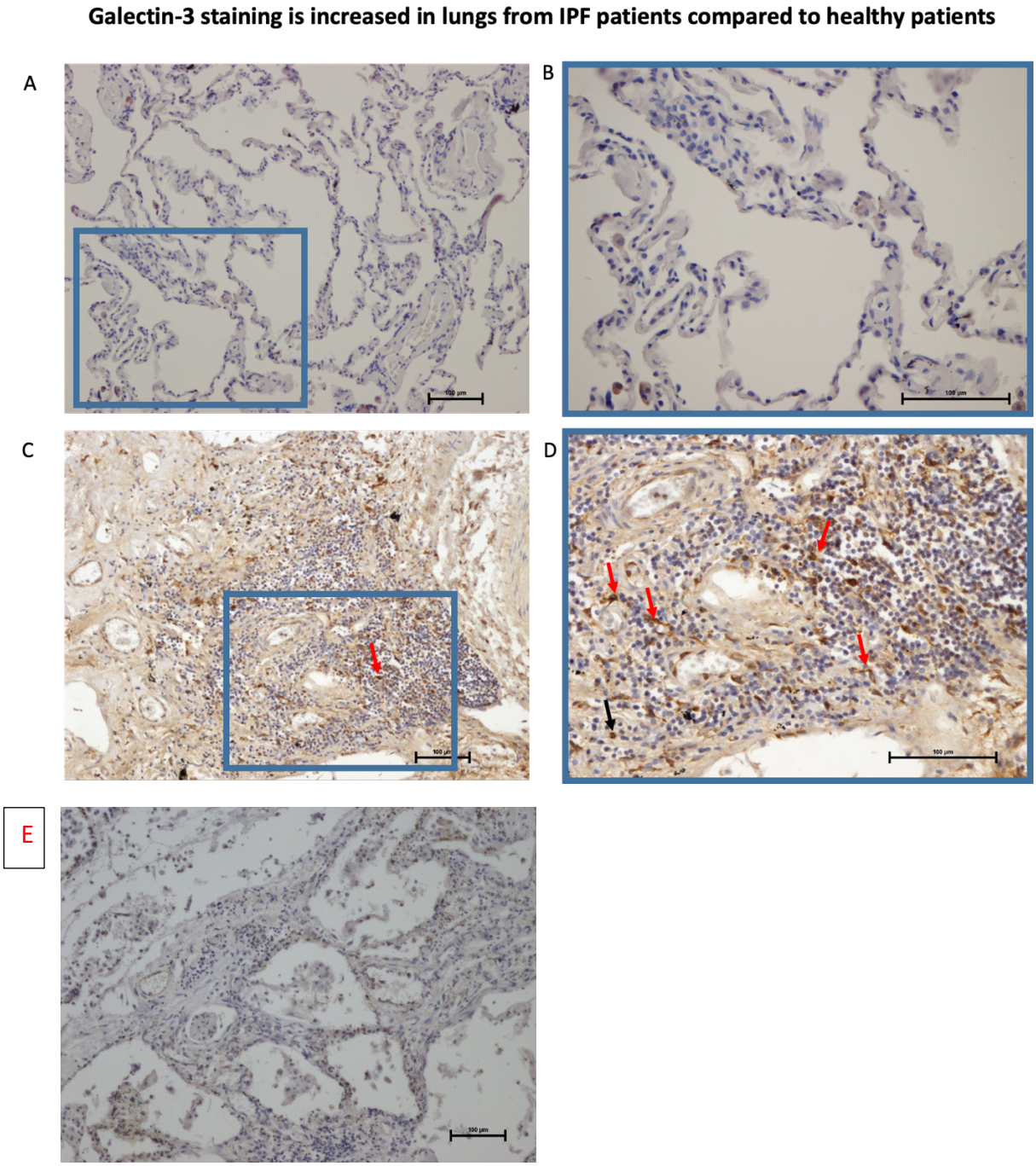



Figure 3. 1

Representative immunohistochemistry images of healthy (A taken at x10 magnification and B taken at x20 magnification) and IPF (C taken at x10 magnification and D taken at x20 magnification) lung sections immuno-stained for galectin-3. Galectin-3 expression is increased in the lungs of IPF patients compared to healthy. E, no secondary antibody control. Expression of galectin-3 is increased in cells that morphologically appear to be macrophages (indicated by black arrows) and fibroblasts (indicated by red arrows) in the lungs of IPF compared to healthy individuals. Representative image from N=3 healthy lung tissue and N=4 from IPF lung tissue.

**Galectin-3 staining in lungs from IPF patients is localised in fibrotic areas**

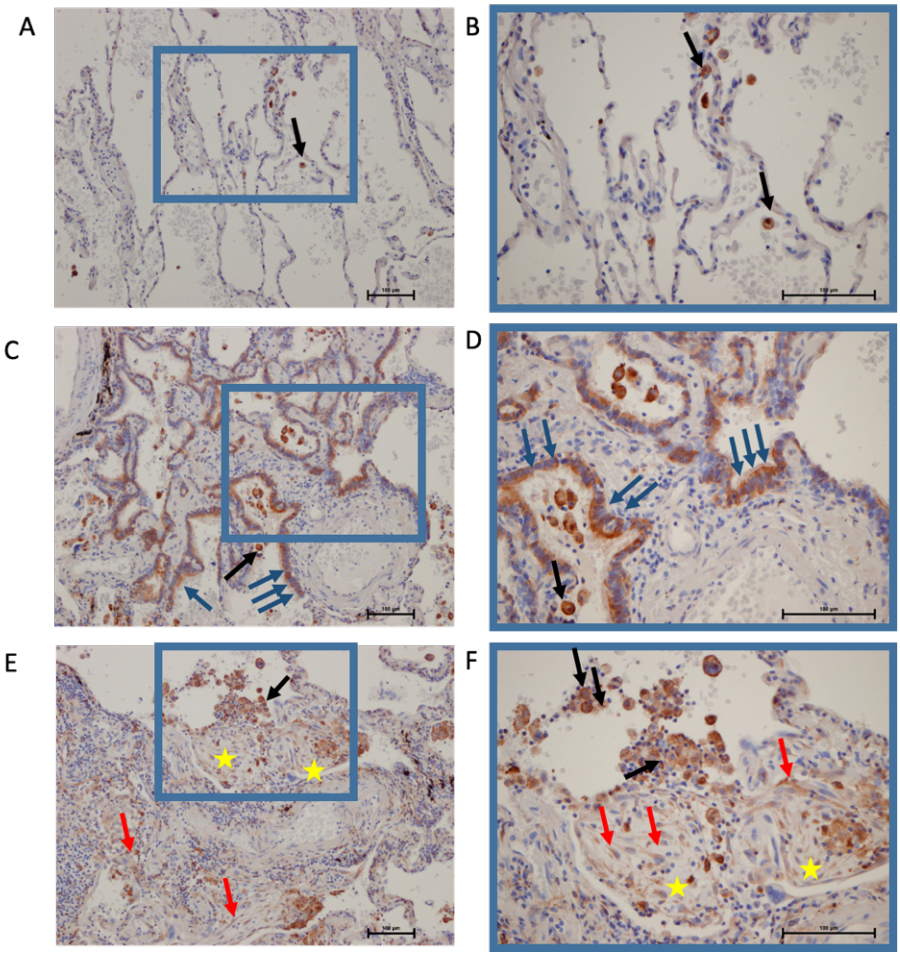



*Figure 3. 2*

Representative immunohistochemistry images of the same IPF lung section immuno-stained for galectin-3. Figures show areas of normal lung from an IPF lung (A taken at x10 magnifcation and B taken at x20 magnification), and areas of fibrosis (C and E taken at x10 magnification and D and F taken at x20 magnification). Galectin-3 expression is increased in the fibrotic areas of the lung compared to the normal areas notably in macrophages (black arrow), epithelial cells (blue arrows) and fibroblasts (red arrows). Yellow star indicates the areas of fibroblast foci. Representative image from n=4.

**Galectin-3 is increased in lungs from bleomycin treated mice**

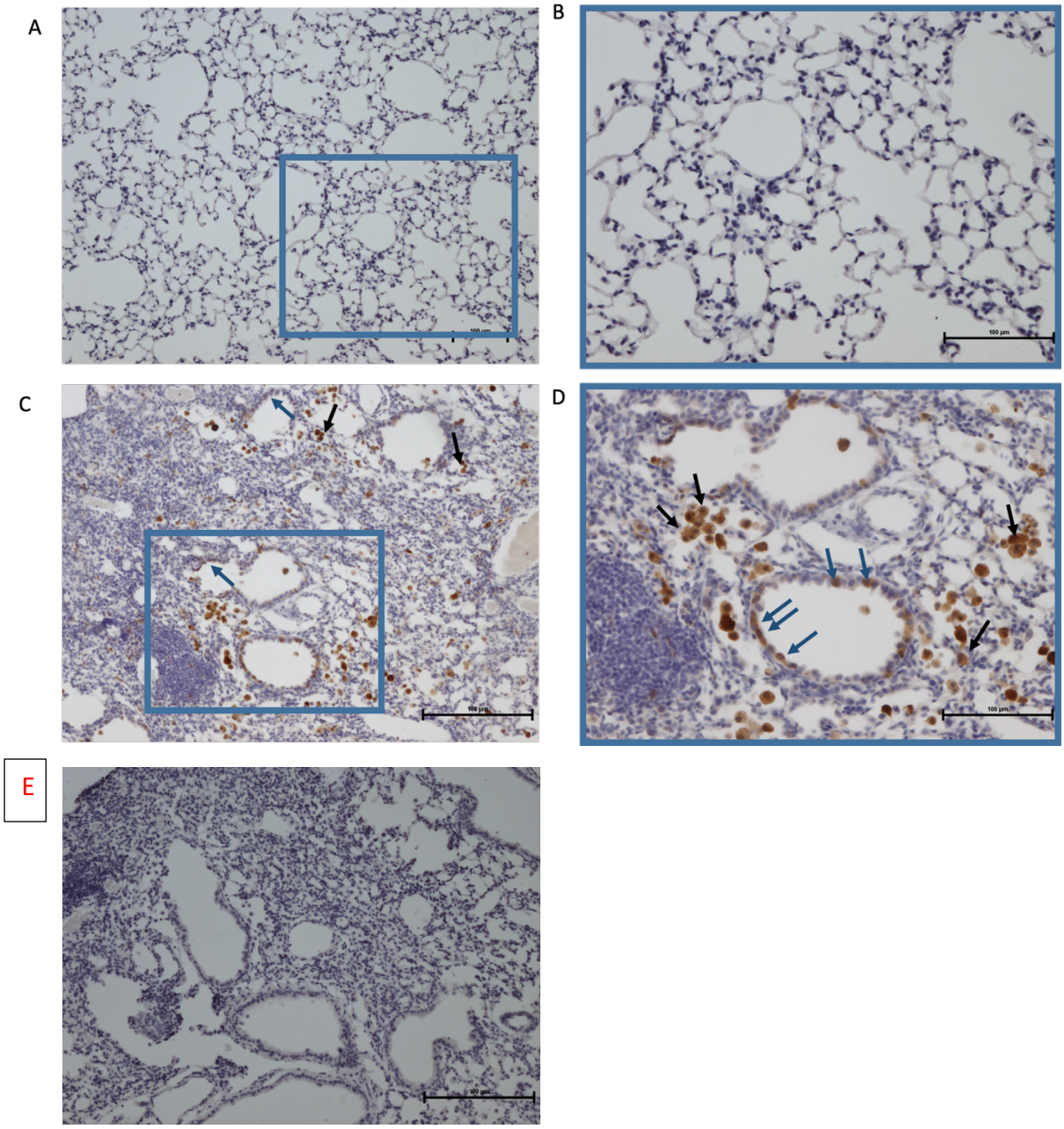



*Figure 3. 3*

Representative immunohistochemistry images of mouse lung sections immuno-stained for galectin-3. C57BL/6 mice were treated with saline (A taken at x10 magnification and B taken at x20 magnification) or bleomycin (C taken at x 10 magnification and D taken at x20 magnification) for 28 days and stained with anti-galectin-3 antibody. E, no secondary antibody control. Galectin-3 is upregulated in bleomycin treated mice where upregulation is observed in epithelial cells (blue arrows) and macrophages (black arrows). Representative images from n=3 from each treatment group.

## Galectin-3 expression and release after TGFβ treatment

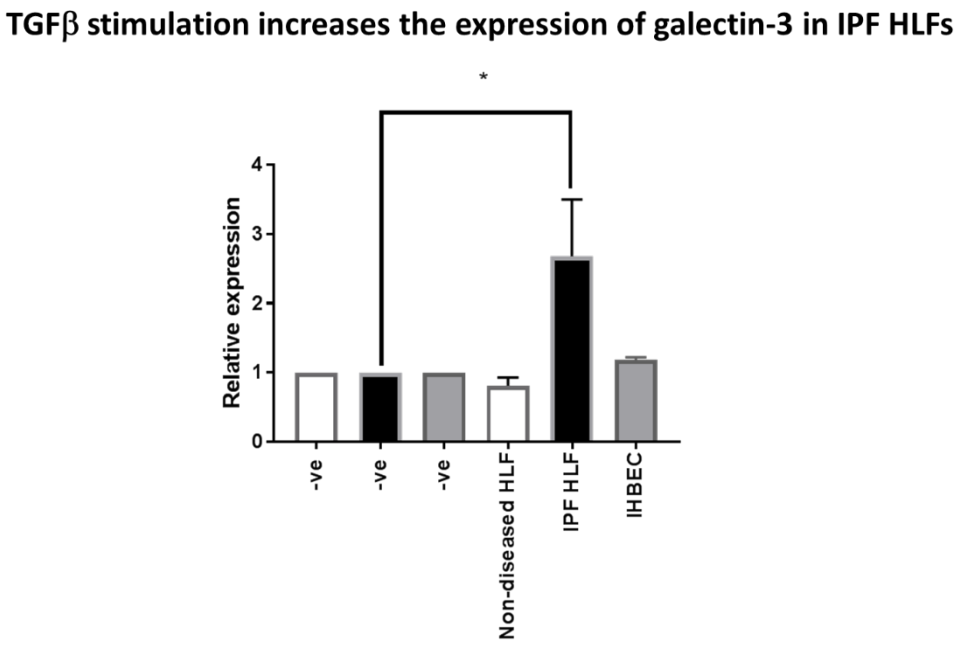



*Figure 3. 4*

Healthy HLFs, IPF HLFs and IHBECs were treated with 2ng/ml TGFb for 24hrs and expression of galectin-3 was investigated by qPCR. Data presented as mean +/- SEM. N=3. *p < 0.05.

I have shown galectin-3 is increased in epithelial cells and fibroblasts in IPF (see figure 3.1 and 3.2) and that bleomycin treatment increased expression of galectin-3 in the lungs of mice with pulmonary fibrosis (figure 3.3). To better understand the effect of TGFβ on the specific cells that express galectin-3, I measured galectin-3 mRNA expression in normal HLFs, IPF HLFs and IHBECs treated with 2ng/ml TGFβ for 24 hours (figure 3.4). TGFβ stimulation of normal HLFs and IHBECs had no change in galectin-3 expression levels. However, galectin-3 expression was significantly increased in IPF HLFs after 24 hours TGFβ stimulation (see figure 3.4).

**TGFβ stimulation has no effect on galectin-3 released by fibroblasts and epithelial cells**

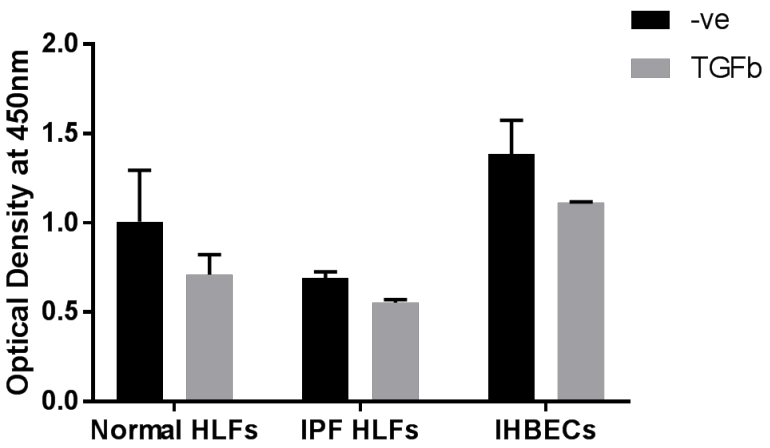



*Figure 3. 5*

Healthy HLFS, IPF HLFs and IHBECs were treated with TGFb for 24hrs and galectin-3 release was investigated by ELISA. Data presented as mean +/- SEM. N=3.

Galectin-3 secretion after TGFβ stimulation was measured by ELISA (figure 3.5). Normal and IPF HLFs as well as IHBECs were treated with 2ng/ml TGFβ for 24 hours and galectin-3 release was measured by ELISA. Results show that after TGFβ there is no change in galectin-3 released by healthy HLFs, IPF HLFs, and IHBECs (see figure 3.5).

The effect of TGFβ stimulation on intracellular galectin-3 expression was investigated by immunocytochemistry (figure 3.6). IPF HLFs were treated with 2ng/ml TGFβ at different time points and were then stained for galectin-3. Results

show that following 2 and 4 hours of TGFβ stimulation there are no visible changes in galectin-3 levels (See figure 3.6B and 3.6D). However, after 48 hours TGFβ treatment the there is a visible decrease in intracellular levels of galectin-3 through observation(figure 3.6F). The reason why there is a decrease in galectin-3 at 48 hours post TGFβ stimulation will need to be investigated further.

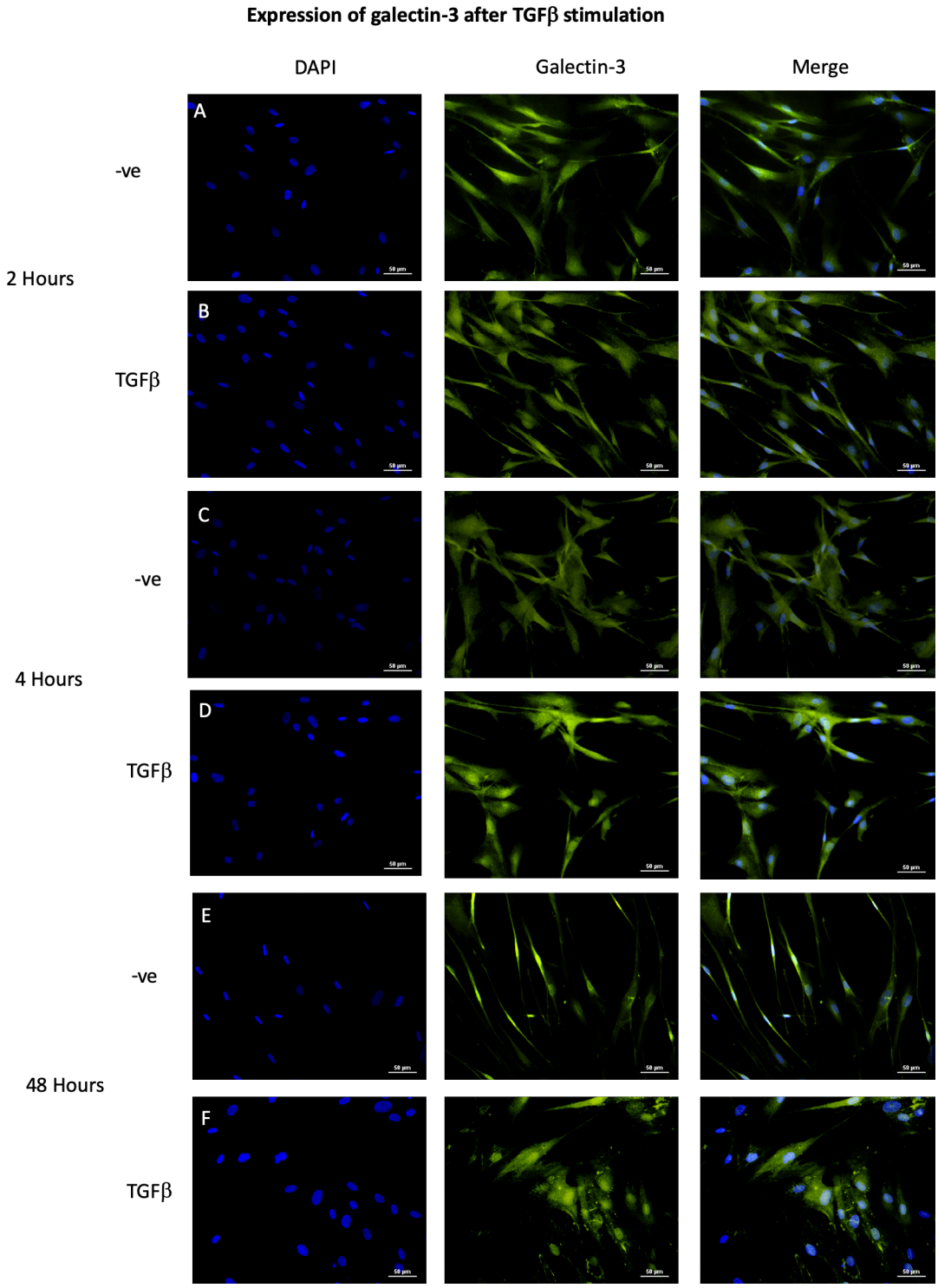



*Figure 3. 6*

IPF HLFs were treated with TGFβ and galectin-3 expression was investigated by immunofluorescence (green). HLFs were plated on an 8-well chamber slide for 24 hours prior to serum starvation. Cells were treated for 2, 4 and 48 hours. Expression of galectin-3 decreased after 48 hours of TGFβ stimulation through observation. Cells were fixed with methanol. Images were acquired by Leica TCS SP2 confocal microscope.

# Discussion

## Summary of findings

Results from this chapter have shown that galectin-3 expression is increased in the lung of patients with IPF compared to control patients. In IPF lungs, this increase in galectin-3 concentration was found spatially in the fibrotic areas and fibroblastic foci. Whereas normal areas of lung tissue in IPF lungs had decreased galectin-3 expression. In human IPF, the increased galectin-3 expression was seen in cells that morphologically appear to be macrophages, fibroblasts and epithelial cells. In bleomycin induced lung fibrosis mouse model, galectin-3 expression was increased compared with saline treated mice controls. This increased galectin-3 expression was seen in cells that morphologically appear to be macrophages and epithelial cells.

Furthermore, TGFβ stimulation increased mRNA expression of galectin-3 in IPF fibroblasts but had no change in expression was seen in normal fibroblasts or epithelial cells. However, no change in galectin-3 secretion at 24 hours was seen in response to TGFβ stimulation in cells. In fibroblasts, the effect of TGFβ on intracellular galectin-3 expression was investigated. Results showed that expression of galectin-3 decreased at 48 hours.

## Galectin-3 expression in IPF

Results from this chapter showing galectin-3 levels are increased in the lungs IPF patients compared with healthy individuals highlight that galectin-3 plays a role in the disease process. It has also been noted that galectin-3 concentrations are increased in the BALF from IPF patients compared to healthy controls (Nishi *et al.*, 2007). Further investigation into this revealed that alveolar macrophages from IPF patients express more galectin-3 than control subjects (Nishi *et al.*, 2007). Interestingly, they found that galectin-3 concentrations were lower in the pulmonary fibrosis patients receiving corticosteroid treatment (Nishi *et al.*, 2007). Additionally, MacKinnon *et al.* 2012 had also found that galectin-3 contractions are increased in IPF patients compared with age matched controls. Furthermore, the serum galectin-3 levels are also increased in the IPF patients (MacKinnon *et al.*, 2012). The serum galectin-3 levels sharply rose during IPF acute exacerbation (MacKinnon *et al.*, 2012).

Kasper and Hughes, 1996, noted that in irradiation induced lung inflammation in rats, galectin-3 expression was increased. The increased galectin-3 levels were seen in interstitial and alveolar macrophages and type 1 alveolar epithelial cells (Kasper and Hughes, 1996). Similarly, I observed that bleomycin increased galectin-3 expression in macrophages and epithelial cells. Interestingly, in irradiation induced lung fibrosis in rats, there was less galectin-3 expression in type 2 alveolar epithelial cells compared to type 1 (Kasper and Hughes, 1996). Wildtype mice treated with TGFβ adenovirus had increased collagen deposition and lung fibrosis (MacKinnon *et al.*, 2012). The increased expression was seen in macrophages and bronchial epithelial cells (MacKinnon *et al.*, 2012). The increased galectin-3 expression was

spatially and temporally related to areas of fibrosis in the TGFβ treated mice (MacKinnon *et al.*, 2012). This is an observation I also made, where in IPF lungs the expression of galectin-3 is related to the areas of fibrosis. Results from this chapter has shown this bleomycin treated mice, another lung fibrosis model, galectin-3 is upregulated in macrophages and epithelial cells. The results therefore show that galectin-3 plays an important role in the pathogenesis of IPF.

## TGFβ and galectin-3

Galectin-3 -/- mice treated with TGFβ adenovirus showed significant decrease in lung fibrosis development (MacKinnon *et al.*, 2012). This highlights that galectin-3 plays an important role in TGFβ mediated fibrosis. Galectin-3 -/- fibroblasts showed a decreased TGFβ induced collagen-1 and αSMA compared to wildtype lung fibroblasts (MacKinnon *et al.*, 2012). Additionally, galectin-3 -/- epithelial cells also showed a decreased in TGFβ mediated responses (MacKinnon *et al.*, 2012). Epithelial cell treated with TGFβ showed an increase in galectin-3 secretion after 72 hours TGFβ treatment (MacKinnon *et al.*, 2012). However, as shown in figure 3.5 this could not be replicated in my study where epithelial cells and fibroblasts treated with TGFβ displayed no changes in galectin-3 secretion. However, this was only after 24 hours TGFβ stimulation.

TGFβ treatment of healthy fibroblasts and epithelial cells showed no changes in galectin-3 expression (figure 3.4). Interestingly however, IPF fibroblasts treated

with TGFβ showed a significant increase in the mRNA expression of galectin-3 (figure 3.4). Indicating that that IPF fibroblasts are more sensitive to TGFβ induced galectin-3 increases. The results could possibly indicate that in healthy fibroblasts, galectin-3 levels are regulated through an undescribed protective mechanism. Thus, this mechanism is lost in IPF, resulting in increased galectin-3 expression and activity contributing to fibrosis development and progression. Furthermore, investigating the effect of TGFβ stimulation on intracellular galectin-3 levels in IPF fibroblasts had shown that there were no changes in galectin-3 levels after 2 and 4 hours of TGFβ treatment. However, after 48 hours of TGFβ treatment, there was visible decrease in the intracellular galectin-3 expression levels. This decrease is thought to be as a result from the galectin-3 secreted from the cell. This however will need to be investigated further. Overall, the work highlights the important role galectin-3 plays in TGFβ signalling pathways and TGFβ induced responses.

## Summary, limitation and future work

In summary, I have shown that galectin-3 expression in the lungs is increased in IPF patients compared with controls. Additionally, I have shown that galectin-3 expression is increased in mice treated with bleomycin compared with control. The increases in galectin-3 expression in diseased lungs and experimentally induced fibrosis were seen in cells that morphologically appear to be macrophages, epithelial cells and fibroblasts.

TGFβ stimulation had no effect on galectin-3 secretion in fibroblasts and epithelial cells at the time point assessed. TGFβ treatment had no effect on the mRNA expression of galectin-3 in healthy fibroblasts and epithelial cells but significantly increased galectin-3 mRNA levels in IPF fibroblasts at 24 hours.

A limitation of my study is the use of immortalized epithelial cells to investigate the effect of TGFβ stimulation on galectin-3 expression. To become immortalized the cells would have to undergone significant mutations which could alter the biology of the cell and therefore do not completely mimic primary cells (Ramirez *et al.*, 2004). Therefore, to offer improved translatable data primary epithelial cell lines should have been used.

Another limitation of the study is that in investigating the effect of TGFβ treatment on intracellular galectin-3 levels timepoints 2, 4 and 48 hours were used. Future work investigating this would not only have more time points (2, 4, 8, 12, 24 and 48 hours) but also compare the intracellular levels of IPF fibroblasts to healthy HLFs and epithelial cells.

Future experiments would explore more timepoints in investigating TGFβ stimulation on galectin-3 mRNA expression and galectin-3 secretion. In my study only the effect at 24 hours was explored. The study conducted by Mackinnon *et al*. 2012 showed an increased in galectin-3 secretion in epithelial cells after 72 hours TGFβ stimulation.

Lastly, future work will investigate the increased galectin-3 mRNA expression after TGFβ treatment demonstrated by IPF HLFs. The work will look at the potential protective mechanism seen in the healthy HLFs and which is lost in IPF fibroblasts resulting in increased galectin-3 mRNA levels and expression which therefore can contribute to IPF disease development.

# Chapter 4: Role of Galectin-3 in TGFβ signalling

# Chapter 4- Role of Galectin-3 in TGFβ signalling

## Introduction

TGFβ signalling is central in the IPF disease pathogenesis. Its action promotes numerous fibrotic pathways. In fibroblasts, TGFβ promotes fibroblasts activation, transformation to myofibroblasts and increased ECM production and deposition (Fernandez and Eickelberg, 2012). However, TGFβ signalling is pivotal in many biological processes such as regulating inflammation, cell differentiation, cell proliferation, tissue homeostasis, and wound healing (Wu and Hill, 2009). It is also an important mediator of tissue growth where is it involved in processes such as angiogenesis and vasculogenesis during embryonic development (Wu and Hill, 2009). Studies using TGFβ deficient mice have shown that these mice develop multiorgan autoimmune inflammatory disease and die within a few weeks after birth (Shull *et al.*, 1992; Kulkarni *et al.*, 1993). In addition, systemic inhibition of TGFβ has led to the development of severe complications such as squamous skin tumours and autoreactive autoimmunity (Mordasky Markell *et al.*, 2010; Akhurst and Hata, 2012; Travis and Sheppard, 2014; Lacouture *et al.*, 2015). Thus, targeting TGFβ activation or signalling in a cell type specific manner could minimise the risk of adverse effects seen with global TGFβ inhibition.

TGFβ expression is increased in IPF tissues compared with healthy lung tissue (Khalil *et al.*, 1996, 2001; Xu *et al.*, 2003). Approximately, 80% of upregulated genes in the lungs of IPF patients are directly or indirectly TGFβ target genes (Naftali Kaminski *et al.*, 2000). In *in vivo* models of pulmonary fibrosis, TGFβ signalling is increased in the bleomycin injury model (Nakao *et al.*, 1999; Yehualaeshet *et al.*, 2000), and overexpression of TGFβ leads to the development of pulmonary fibrosis *in vivo* (Sime *et al.*, 1997; Lee *et al.*, 2018).

TGFβ exerts its cellular effects by activating serine/threonine receptors such as TGFβ receptor I (TGFβRI/ Alk5) and TGFβ receptor II (TGFβRII). TGFβ binding to TGFβRII results in a complex formation with TGFβR1 (Wrana *et al.*, 1992; Anders and Leof, 1996), consequently this leads to the activation of a downstream signalling cascade. TGFβ primarily signals through the canonical Smad signalling pathway (Nakao *et al.*, 1997; Feng and Derynck, 2005; Hill, 2009; Macias, Martin-Malpartida and Massagué, 2015), however, non-canonical signalling pathways are also observed (Derynck and Zhang, 2003; Moustakas and Heldin, 2005). Once phosphorylated by ligation of the TGFβ receptor, Smad2 or Smad3 complexes with Smad4. The Smad2/Smad4 or Smad3/Smad4 complex then translocates to the nucleus to regulate gene transcription. Non-canonical (non-Smad2) pathways can also be activated by TGFβ, such as PI3K/AKT (Zhang, Zhou and ten Dijke, 2013; Bauer *et al.*, 2015), MAPK/ERK (Chapnick *et al.*, 2011; Bauer *et al.*, 2015), Wnt (Piersma, Bank and Boersema, 2015), and notch pathways (Fu *et al.*, 2009). The non-canonical

TGFβ signalling pathway has been shown to be important in IPF disease pathways such as myofibroblast activation (Sousa *et al.*, 2007).

The expression of galectin-3 has shown to be increased in fibrosis (Henderson *et al.*, 2006, 2008; MacKinnon *et al.*, 2012). Furthermore, galectin-3 knockout mice are protected from TGFβ overexpression induced pulmonary fibrosis (MacKinnon *et al.*, 2012). In addition, wild type mice where TGFβ was overexpressed had an increased galectin-3 expression in macrophages, bronchial epithelium, and the BALF (MacKinnon *et al.*, 2012). Importantly, Mackinnon e*t al*. (MacKinnon *et al.*, 2012) showed that there was no difference between the expression of active TGFβ in wildtype mice and galectin-3 -/- mice, indicating that galectin-3 somehow regulates TGFβ activity. The knockout of galectin-3 in epithelial cells resulted in a reduction of TGFβ receptor expression at the cell surface (MacKinnon *et al.*, 2012). The study also demonstrated that inhibition of galectin-3 with the small-molecule inhibitor TD139 attenuated bleomycin induced lung fibrosis by dramatically decreasing collagen deposition (MacKinnon *et al.*, 2012).

## Aims and Objectives

In the previous chapter I have shown that galectin-3 expression is increased in IPF and in *in vivo* models of pulmonary fibrosis. In my introduction I describe the evidence to support the concept that galactin-3 can mediate fibrosis via TGFβ signalling. Anti-galectin-3 treatments are currently in clinical trials for IPF treatment.

However, the mechanisms through which galectin-3 leads to TGFβ signalling are poorly understood. It is hypothesised that galectin-3 plays a vital role in the TGFβ signalling pathway. Thus, the aim of this chapter are:

1. Investigate the effect of galectin-3 inhibition on TGFβ signalling in fibroblasts and epithelial cells.
2. Investigate the effect of galectin-3 stimulation on TGFβ signalling in fibroblasts and epithelial cells.

# Methods

## Specific assays

The role of galectin-3 on TGFβ signalling was investigated with the use of the galectin-3 inhibitors and their effect on TGFβ stimulated Smad2 phosphorylation. The changes in psmad2 levels was analysed by western blot.

MEFβ6, human lung fibroblasts (HLFs) from healthy (n=3) and IPF patients (n=3), and immortalised bronchial epithelial cells (IHBECs) were utilised in this study. Cells were grown until ~80% confluent and DMEM was changed to serum free DMEM. After 24 hours of serum starvation the cells were treated with (0.1µM-10µM) of galectin-3 inhibitors (GB1211, GB1107, TD139), 50µM of Alk5 inhibitor (positive inhibitory control), or equivalent volume of DMSO (negative control). After 20 minutes of galectin-3 inhibitor or Alk5 inhibitor pre-incubation the cells were treated with 2ng/ml TGFβ or 10µg/ml recombinant human galectin-3 and incubated for 2 hours. After the 2 hours of incubation, cells were lysed, scrapped, and stored at -80$^{o}$C to await analysis. The concentration of the cell lysates was determined via BCA assay.

## Western blot

Proteins were separated via SDS-PAGE. 15-25µg of protein was loaded per lane in a 10% SDS-polyacrylamide gel. After electrophoresis stage of the western blot,

protein was transferred from the gel to a polyvinylidene difluoride membrane (PVDF). After the transfer stage, the PVDF membranes were blocked with 5% milk (5% non-fat milk in TBS plus 0.1% Tween 20) for 1 hour at room temperature. The membranes were then incubated overnight at 4$^{o}$C with anti-Psmad2 primary antibody (Phospho-Smad2 (Ser465/467) (138D4) Rabbit mAb #3108, Cell Signalling Technology) at 1:1000 dilution in blocking buffer. Following overnight primary antibody incubation membranes were washed with TBST and then incubated with HRP-conjugated anti-rabbit secondary antibody (1:3000 dilution in blocking buffer) for 1 hour at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL.

Following visualisation of Psmad2, membranes were probed further for anti-Glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) (1:10000 dilution in blocking buffer) for 1-hour room temperature. Membranes were washed in TBST and then be incubated with HRP-conjugated anti-rabbit secondary antibody (1:3000 dilution in blocking buffer) for 30mins at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL. GAPDH was used as the loading control.

Following visualisation of GAPDH, membranes were stripped using stripping buffer for 5 minutes. Membranes were washed in TBST and blocked with 5% milk (5% non-fat milk in TBS plus 0.1% Tween 20) for 1 hour at room temperature. The membranes were then incubated overnight at 4$^{o}$C with anti-smad2/3 primary

antibody (smad2/3 Rabbit polyclonal Ab #3102, Cell Signalling Technology) at 1:1000 dilution in blocking buffer. Following overnight primary antibody incubation membranes were washed with TBST and then incubated with HRP-conjugated anti-rabbit secondary antibody (1:3000 dilution in blocking buffer) for 1 hour at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL.

The western blot bands were quantified using ImageJ software. Results are expressed as a band density ratio of pSmad2 to Smad2, which indicates the relative expression of pSmad2.

## TGFβ reporter assay

The role of galectin-3 on TGFβ activity was investigated with the use of recombinant human galectin-3 and its effect on TGFβ activity in a TMLC assay. TMLCs were seeded at a density of $5x10^4$ cells/ml to measure the total active TGFβ. Recombinant human galectin-3 was added in a concentration dependent manner (2.5µg/ml-20µg/ml). Recombinant human TGFβ1 was added to relevant wells in a concentration dependent manner (62.5pg/ml-5000pg/ml). TGFβ1 treatments was used as a standard curve. After 16h incubation at 37$^{o}$C, cells were washed in PBS and lysed with 50µl of reported lysis buffer (Promega). The lysates were then transferred to a luminometry plate, 100µl of luciferase reagent was added and the luciferase activity was immediately measured using FLOUstar omega multi-mode microplate

reader. All experiments were performed in triplicates and was repeated at least three times.

## Flowcytometry

Cells were grown in T150 flasks until approximately 80% confluent. Media was aspirated from cells and cells were trypsinised and counted then adjusted to 100,000 cells in 100µl PBS and sodium azide. Non-specific binding was blocked in 5% goat serum for 30mins. CD98 primary antibody (abcam, ab2528) at 5µg/ml was added to cells and incubated for 30mins at 4$^{o}$C. 1ml PBS and sodium azide was then added to wash the cells, centrifuged and supernatants removed. The cellular pellet was resuspended in 200µl PBS plus sodium azide. Secondary goat anti-mouse antibody conjugated with phycoerythrin (New England Bio Labs) was added (1:200 dilution) and incubated for 30mins at 4$^{o}$C. Cells were again washed with PBS, centrifuged and responded in PBS and sodium azide. The fluorescence was then quantified via mean fluorescence intensity on 100,000 cells with FacsCalibur with FlowJo version 10.1 software.

# Results

## Effect of galectin-3 inhibition on TGFβ activity

**Effect of Galectin-3 inhibitors on Smad2 phosphorylation in TGFβ stimulated Mouse embryonic fibroblast cells expressing the integrin β6 (MEFβ6)**

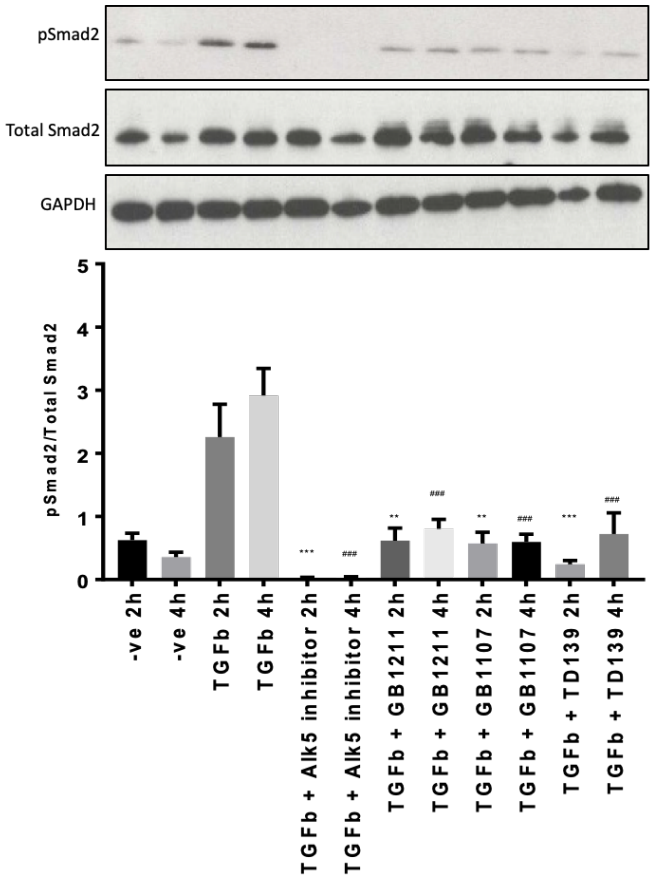



*Figure 4. 1*

Effect of galectin-3 inhibition on TGFβ signalling in mouse embryonic fibroblasts expressing integrin β6 (MEFβ6). MEFβ6s were treated with TGFβ for 2 and 4 hours with 3 different galectin-3 inhibitors that were pre-treated for 20mins prior to TGFβ stimulation. Cells lysates were harvested after time points and western blot was run to measure levels of TGFβ activity via pSmad2 levels. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. ** p<0.01 and ***p<0.001 as compared to cells treated with TGFβ 2hours. ### p<0.001 as compared to cells treated with TGFβ 4hours N=3.

**Effect of galectin-3 inhibition on TGFβ signalling in human lung fibroblasts (HLFs) and epithelial cells**

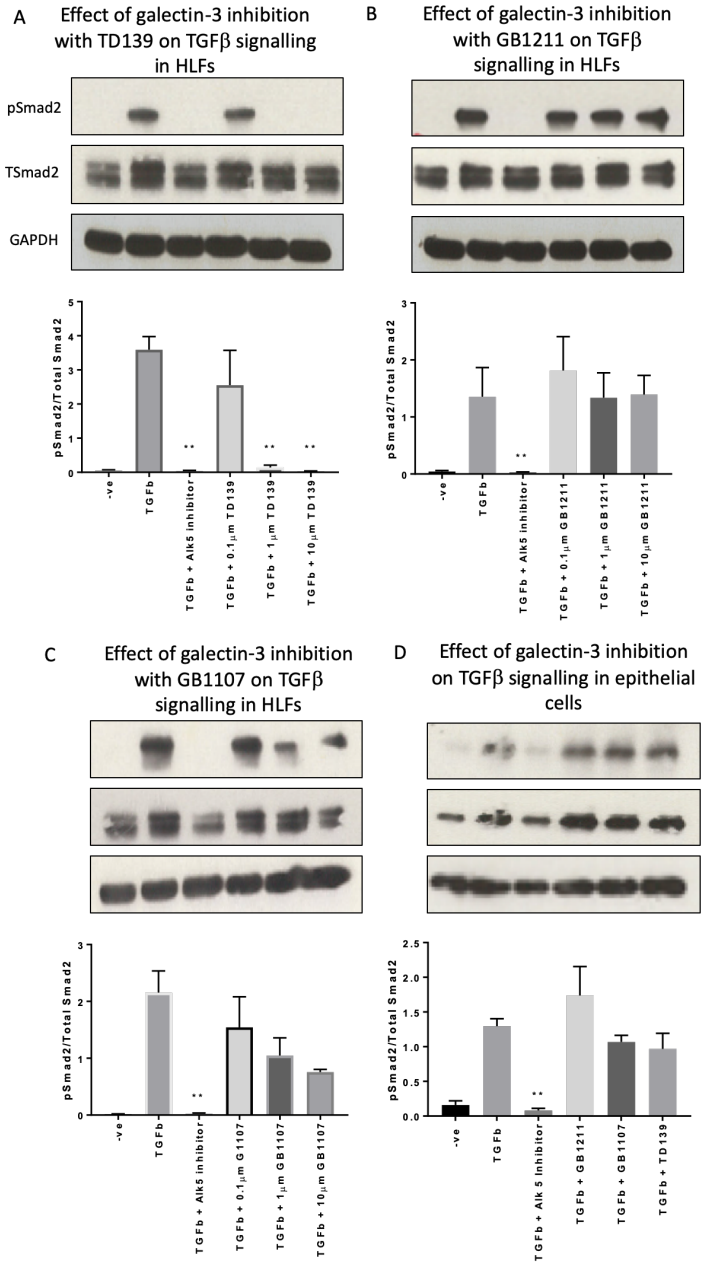



*Figure 4. 2*

Effect of galectin-3 inhibition on TGFβ signalling in human lung fibroblasts (HLFs) (A-C) and immortalised human bronchial epithelial cells (IHBECs) (D). Cells were treated with 2ng/ml TGFβ for 2 hours and pre-treated with galectin-3 inhibitors. (A) TD139, (B) GB1211, (C) GB1107. Cells lysates were harvested after time points and western blot was run to measure levels of TGFβ activity via pSmad2 levels. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. ** $p<0.01$ as compared to cells treated with TGFβ (+ve). N=3.

TGFβ signalling promotes numerous fibrotic pathways in fibroblasts such as fibroblast activation, transformation to myofibroblast, and increased ECM synthesis. Mouse embryonic fibroblasts expressing the integrin β6 (MEFβ6) were pre-treated with 3 different galectin-3 inhibitors (GB1211, GB1107, TD139) at a 1μM. GB1211 and GB1107 are similar in structure and specific to galectin-3. The drug TD139 is currently in clinical trials for IPF treatment. TD139 has high affinity for both galectin-3 (Kd 14nM) and galectin-1 (Kd 10nM). TGFβ treatment increased Smad2 phosphorylation after 2 hours and further increased pSmad2 levels after 4 hours of treatment (figure 4.1). The TGFβ mediated pSmad2 increases were significantly blocked by inhibiting TGFβ receptor 1 (ALK5) at both 2 and 4 hours. Pre-treatment with galectin-3 inhibitor GB1211 significantly decreased TGFβ-induced pSmad2 increases at 2 and 4 hours. GB1107 also significantly reduced smad2 phosphorylation mediated by TGFβ at 2 and 4 hours. Inhibition of galectin-3 and galectin-1 by TD139 significantly attenuated TGFβ-dependent smad2 phosphorylation again at 2 and 4 hours.

To explore the role of galectin-3 in human cells, I investigated the effect of galectin-3 inhibition on TGFβ signalling in human lung fibroblasts (HLFs) and immortalised human bronchial epithelial cells (iHBECs) (figure 4.2). HLFs were treated with 2ng/ml TGFβ and galectin-3 action was block via pre-treatment of the 3 anti-galectin-3 compounds: TD139 (figure 4.2A), GB1211 (figure 4.2B) and GB1107 (figure 4.2C). TGFβ treatment increased Smad2 phosphorylation, which was blocked by ALK5 inhibition (figure 4.2A-D). Inhibition of galectin-3 and galectin-1 with

compound TD139 significantly attenuated TGFβ mediated pSmad2 increases at both 1µM and 10µM with an IC50 of approximately 0.55µM (figure 4.2A). Interestingly, GB1211 showed no effect of pSmad2 levels at the different concentrations (figure 4.2B). However, galectin-3 inhibitor GB1107 did have a dose dependent decrease in TGFβ mediated smad2 phosphorylation, although this did not reach the significance threshold (figure 4.2C).

The effect of galectin-3 inhibition on TGFβ signalling in epithelial cells was also explored (figure 4.2D). TGFβ stimulation in IHBECs increased pSmad2 levels as expected. This increase in TGFβ signalling was blocked by inhibition of TGFβR1 (Alk5). Interestingly, unlike in the fibroblasts, galectin-3 inhibition with compounds GB1211, GB1107 and TD139 at 1µM had no effect pSmad2 levels. Results indicate that galectin-3 plays an important role in the TGFβ signalling pathway in fibroblasts but not in epithelial cells.

**The effect of galectin-3 inhibition on TGFβ treated human lung fibroblasts from healthy patients and IPF patients**

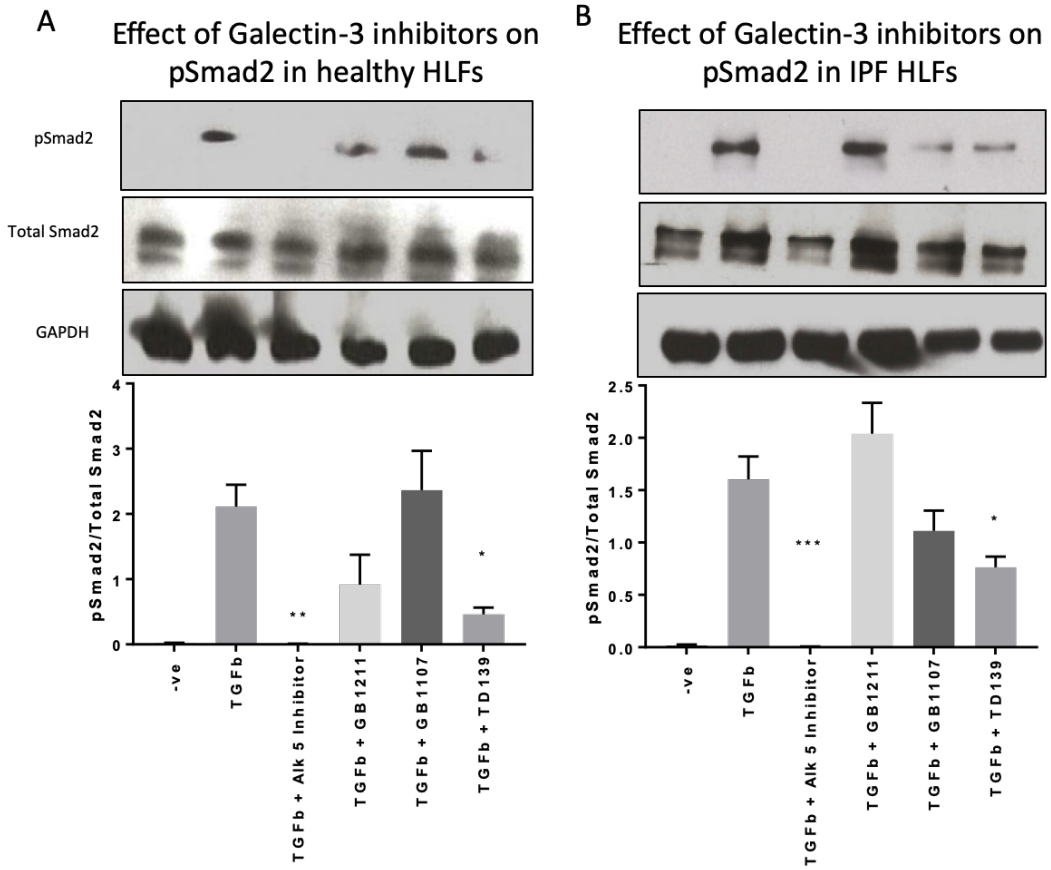



*Figure 4. 3*

Effect of galectin-3 inhibition on TGFβ signalling in fibroblasts derived from healthy patients (A) vs fibroblasts derived from IPF patients (B). Cells were treated with 2ng/ml TGFβ for 2 hours and pre-treated with galectin-3 inhibitors 1μM TD139, 1μM GB1211, 1μM GB1107. Cells lysates were harvested after time points and western blot was run to measure levels of TGFβ activity via pSmad2 levels. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. * $p<0.05$, ** $p<0.01$, $p<0.001$ as compared to cells treated with TGFβ. N=3.

To investigate the role of galectin-3 in fibrotic disease I undertook comparative biology experiments in IPF derived fibroblasts compared with control fibroblasts (figure 4.3). TGFβ stimulation of IPF and control fibroblasts increased the levels of Smad2 phosphorylation in both cell types to a similar level. This TGFβ mediated increase in pSmad2 was completely inhibited by blocking Alk5 receptor in both healthy and IPF HLFs (figure 4.3A, 4.3B). TD139 inhibited also inhibited pSmad2 in both control and IPF fibroblasts. GB1211 and GB1107 had inconsistent effects on pSmad2 responses to TGFb in control and IPF fibroblasts (Figure 4.3A and 4.B).

Interestingly, unlike in healthy HLFs, GB1211 had no effect on TGFβ dependent smad2 phosphorylation in IPF HLFs (figure 4.3B). In accordance to the healthy HLF data, GB1107 had no effect on psmad2 levels in IPF HLFs. The inhibition of galectin-3 and galectin-1 with TD139 however significantly decreased the levels of smad2 phosphorylation induced by TGFβ. The result indicate that the galectin-3 inhibitor can block TGFβ signalling in IPF disease. However, the reason why compound GB1211 blocks TGFβ signalling in healthy fibroblasts but not in IPF fibroblasts will need to be investigated further.

## Effect of galectin-3 on TGFβ activity

Having demonstrated that inhibiting galectin-3 can suppress TGFb induced signalling in fibroblasts I investigated whether galectin-3 could directly promote TGFβ signalling in fibroblasts.

Transformed mink lung epithelial cells (TMLCs) reporter cells, stably expressing firefly luciferase driven by the plasminogen activator inhibitor-1 (PAI-1) reporter were used initially. Stimulation of TMLCs with increasing concentrations of recombinant galectin-3, increased the luciferase activity in a concentration dependent manner (figure 4.4A).

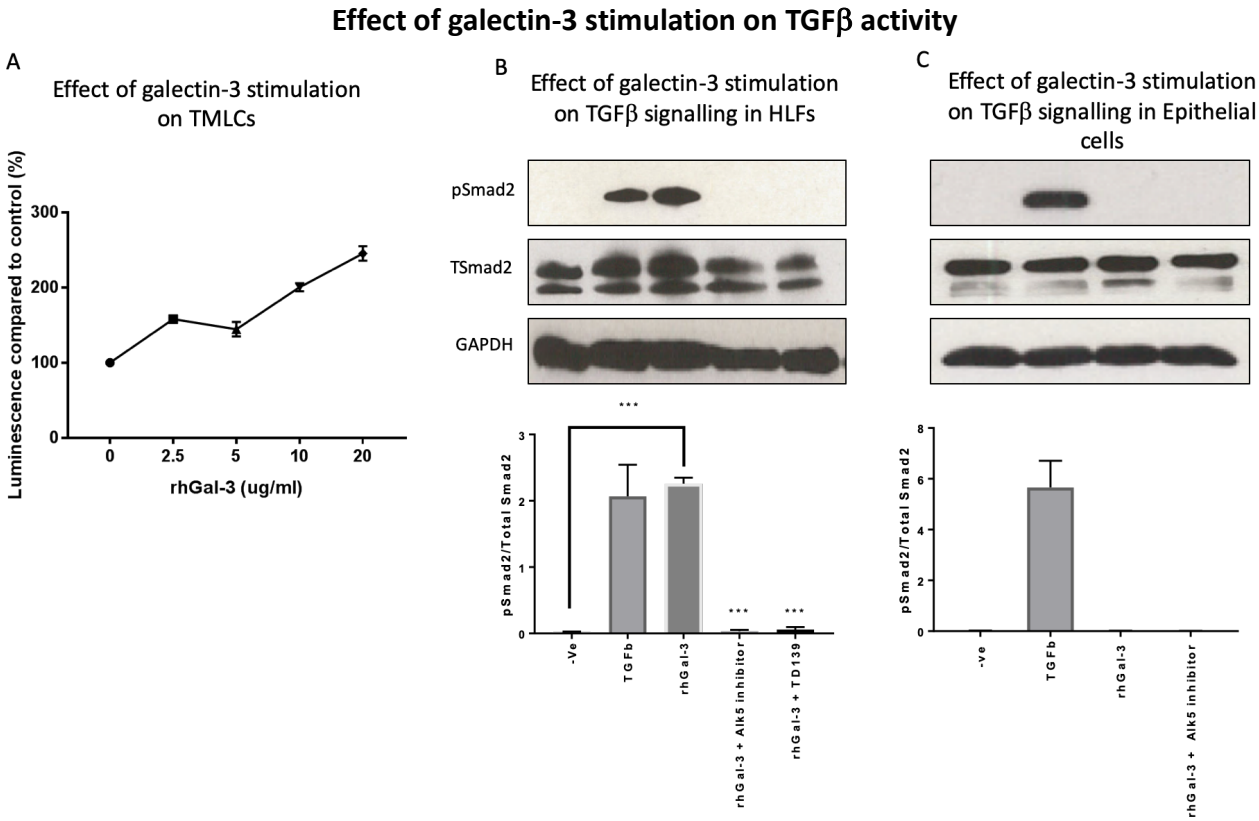



*Figure 4. 4*

Effect of galectin-3 on TGFβ activity in transformed mink lung epithelial cells (TMLCs), HLFs and epithelial cells. TMLCs expressing the luciferase gene at the PAI-1 promoter region were treated with increasing concentrations of rhGal-3 (A). B and C, HLFs and Epithelial cells were stimulated with 10ug/ml rhGal-3. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. *** $p<0.001$ as compared to cells treated with only TGFβ (+ve). N=3.

To confirm that galectin-3 can directly stimulate TGFβ signalling, I stimulated human lung fibroblasts with 10µg/ml galectin-3 and measured pSmad2. Stimulated HLFs responded by increased pSmad2, which was completely inhibited by the TGFβ receptor 1 inhibitor (Alk5 inhibitor) or the galectin-3 inhibitor TD139 (figure 4.4B). TGFβ stimulation, which was used as a positive control, also increased pSmad2 as expected. Galectin-3 stimulation increased levels of pSmad2 to those observed

with TGFβ stimulation. The data indicates that galectin-3 promotes smad2 phosphorylation through either promoting TGFβ activity or directly acting at the TGFβ receptor.

In contrast stimulation of epithelial cells (iHBECs) with 10µg/ml recombinant human galectin-3 did not induce Smad2 phosphorylation whereas stimulation with the positive control TGFβ did induce phosphorylation of smad2 (figure 4.4C).

## Expression of the galectin-3 binding protein- CD98

To understand the differential effects of galectin-3 on epithelial cells and fibroblasts I measured expression of the known galectin-3 binding protein CD98 by flow cytometry (figure 4.5). Epithelial cells expressed high levels of CD98 (figure 4.4A), whereas HLFs did not express the CD98 protein.

**Expression of galectin-3 receptor CD98 in epithelial cells and fibroblasts**

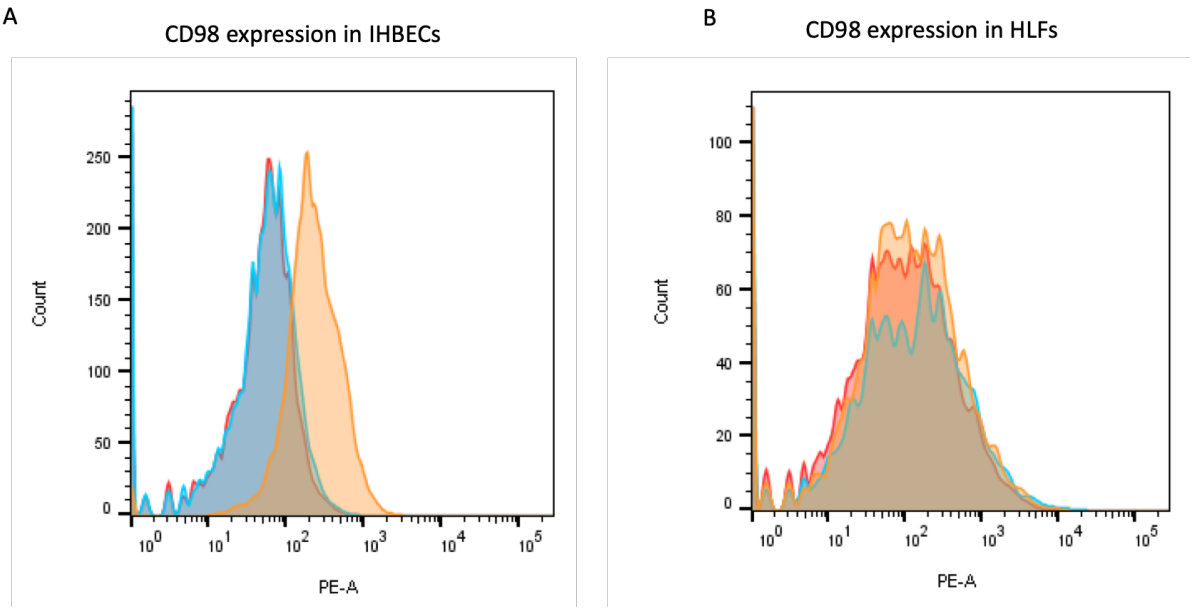



*Figure 4. 5*

Expression of galectin-3 receptor CD98 in (A) IHBECs and (B) HLFs was investigated by flow cytometry. Fluorescence was then quantified via mean fluorescence intensity on 100,000 cells with FacsCalibur with FlowJo version 10.1 software.

# Discussion

## Summary of findings

TGFβ signaling is a crucial pathway in pulmonary fibrosis development, where it is central to fibrotic changes leading to epithelial cell apoptosis, EMT, fibroblast activation, fibroblast transformation to myofibroblasts, and increased ECM synthesis. Results from this chapter show that by inhibiting galectin-3 in mouse and human fibroblasts TGFβ signalling can also be inhibited, and stimulation of fibroblasts with galectin-3 led to an increase in TGFβ signaling. These data suggest that galectin-3 plays a role in the TGFβ signalling pathway. Further studies in diseased cells confirmed that the galectin-3 and galectin-1 inhibitor TD139 consistently inhibited TGFβ signaling, whereas GB1107 and GB1211 had inconsistent effects.

However, galectin-3 stimulation or inhibition had no effect on TGFβ signaling in epithelial cells. This highlights that galectin-3 plays different roles in fibroblasts and epithelial cells, which may be due to differences in galectin-3 receptor expression as observed by CD98 expression.

## Galectin-3 inhibition on TGFβ signaling

TGFβ signalling through the TGFβ receptor complex TGFβRI and TGFβRII, is important to wound repair. Moreover, exaggerated TGFβ signalling is associated with

numerous fibrotic diseases involving the lungs, heart, liver and kidneys (Sheppard, 2006; Hu *et al.*, 2008; Han *et al.*, 2016). In IPF, approximately 80% of upregulated genes are direct or indirect TGFβ target genes (N Kaminski *et al.*, 2000). However, systemic TGFβ inhibition has been shown to lead to the development of tumors and chronic inflammation (Mordasky Markell *et al.*, 2010; Travis and Sheppard, 2014). Therefore, blocking TGFβ signalling through specific cell types or pathways should minimise any adverse effects.

Galectin-3 and galectin-1 inhibition decreased TGFβ signaling in fibroblasts but not in epithelial cells. The importance of galectin-3 in TGFβ has been highlighted in previous studies (Henderson *et al.*, 2006, 2008; MacKinnon *et al.*, 2012). Galectin-3 knock out mice were protected from TGFβ induced pulmonary fibrosis (MacKinnon *et al.*, 2012). Galectin-3 -/- had no effect on TGFβ expression in mouse models (Henderson *et al.*, 2006, 2008; MacKinnon *et al.*, 2012). The knock-out of galectin-3 in the mice had no effect on the increases in total active TGFβ level increases compared to WT mice treated with TGFβ overexpression, indicating that galectin-3 is involved in TGFβ mediated pulmonary fibrosis (MacKinnon *et al.*, 2012). Galectin-3 knock-out lung fibroblasts showed reduced responses to TGFβ stimulation through reduced $\alpha$-SMA and collagen-1 expression after TGFβ stimulation compared to WT lung fibroblasts (MacKinnon *et al.*, 2012). Galectin-3 knock-out epithelial cell also showed less responses to TGFβ stimulation through decreased TGFβ induced EMT compared to WT airway epithelial cells. Interestingly, removal of galectin-3 resulted in the reduction in TGFβ receptor expression at the cell surface. This reduction in

TGFβ receptor expression was rescued by the addition of recombinant human galectin-3 indicating that galectin-3 regulates the expression of TGFβ receptors at the cell surface (MacKinnon *et al.*, 2012). Galectin-3 binds to the polylactosamine residues on the TGFβ receptor, therefore a loss of galectin-3 would result in a decrease in galectin-3 binding to the TGFβ receptors and therefore a decrease in TGFβ receptor retention (Partridge, 2004). This is depicted in figure 4.6.

Our results had shown in HLFs, TD139 decreased TGFβ signaling (as shown in figure 6). However, GB1107 and GB1211 had a lesser effect. GB1107 and GB1211 are highly selective for galectin-3 (Kd 27nM and 23nM respectively and greater than 1000-fold more selective over galectin-1,8 and 9). Whereas, TD139 has a strong affinity for galectin-3 (Kd 14nM) and galectin-1 (Kd 10nM) (MacKinnon *et al.*, 2012). The data therefore demonstrates that galectin-3 is crucial in the TGFβ signaling pathway. The data also shows that galectin-1 may play a key role in TGFβ signaling, however this will need to be investigated further.

Like galectin-3, galectin-1 is localized in the nucleus, cytoplasm, cell surface and secreted into the extracellular spaces. Galectin-1 has a wide variety of functions such as cell proliferation, migration, adhesion, inflammation and apoptosis. The role of galectin-3 in pulmonary fibrosis has been demonstrated by Lim *et al.* (2014) where TGFβ stimulation increased galectin-1 levels in fibroblasts and galectin-1 levels are increased in bleomycin treated mice. The levels of galectin-1 are also increased in the lung of patients with IPF (Kathiriya *et al.*, 2017). Lim *et al*. (2014) had shown that

galectin-1 knockdown led to a decrease in TGFβ induced pSmad2 levels and increased smad2 nuclear retention in fibroblasts. Furthermore, their results showed that galectin-1 directly binds to smad2 and pSmad2 to increase nuclear retention and therefore regulate fibrotic gene expression (Jin Lim *et al.*, 2014). Highlighting that galectin-1 works intracellularly within the TGFβ signaling pathway.

it is postulated that the inhibition of galectin-3 and galectin-1 by TD139 led to the reduction of TGFβ signaling in fibroblasts. This is thought to be through the reduction of galectin-3 binding to the TGFβ receptors leading the rapid removal of TGFβ receptors at the cell surface (Partridge, 2004). This rapid removal of TGFβ receptors at the cell surface reduces TGFβ ligand-TGFβ receptor binding. In synergy with acting with galectin-3, in is hypothesized that TD139 also enters the cell and blocks galectin-1 action and inturn inhibits smad2 activity and phosphorylation. This will need to be investigated further.

My observation that galectin-3 inhibition had no effect on TGFβ induced pSmad2 levels in epithelial cells supports previous observations where knockout of galectin-3 in epithelial cells had no effect on TGFβ induced pSmad2 levels (MacKinnon *et al.*, 2012). However, galectin-3 -/- epithelial cells were able to respond to TGFβ through non-canonical pathways with a reduction in β-catenin activation. The authors showed that TGFβ stimulation of epithelial cells led to β-catenin translocation to the nucleus, whereas in galectin-3 -/- epithelial cells β-catenin expression is maintained at the cell surface. Thus, galectin-3 regulates TGFβ signaling

in epithelial cells through the non-canonical TGFβ signaling pathway. MacKinnon *et al.* (2012) had also shown that galectin-3 knockout mice treated with TGFβ had a reduced β-catenin activation compared to wild-type mice treated with TGFβ. Furthermore, bleomycin treated mice which were administered the galectin-3 inhibitor TD139 had a reduction in lung fibrosis accompanied with a reduction in β-catenin activation (MacKinnon *et al.*, 2012).

TGFβ also plays an important role in the development of renal fibrosis. Galectin-3 -/- mice experimentally induced with progressive renal fibrosis showed no difference in levels of pSmad2 compared to WT mice (Henderson *et al.*, 2008). Furthermore in liver fibrosis, studies have shown that galectin-3 is required for hepatic stellate cell activation and collagen production (Henderson *et al.*, 2006). This highlights the importance of galectin-3 in the TGFβ signaling pathway in fibrotic diseases.

The importance of galectin-3 in the TGFβ signaling pathway was also displayed in intervertebral disc degeneration. Cells of the nucleus pulposus secrete ECM protein during regeneration. It was shown that the expression of galectin-3 in nucleus pulposus cells was regulated in a TGFβ-smad3 dependent manner (Tian *et al.*, 2016). The study provides evidence of the role of galectin-3 in the canonical TGFβ signaling pathway.

When comparing galectin-3 inhibition in fibroblasts from normal and IPF patients it was seen only TD139 had a significant effect on pSmad2 levels. The specific galectin-3 inhibitors GB1107 and GB1211 showed no consistent effects on TGFβ signaling in both normal and IPF fibroblasts. This may reveal that there is no difference in galectin-3 activity between healthy fibroblasts and IPF fibroblasts, however more studies will be required to be definitive.

Direct global TGFβ inhibition has shown to cause high levels of toxic effects. Therefore, to limit the toxicity TGFβ plays critical roles in suppressing inflammation, and systemic inhibition of TGFβ can lead to the development of squamous skin tumors and autoreactive immunity (Mordasky Markell *et al.*, 2010; Travis and Sheppard, 2014). Furthermore, the administration of TGFβRI inhibitors has led to enhanced skin and colonic inflammation and cardiac valve abnormalities (Anderton *et al.*, 2011; Lahn *et al.*, 2015). Thus, to minimize the adverse effects of TGFβ inhibition cell specific targets and pathways need to be targeted. My data has shown that the decrease in canonical TGFβ signaling was mediated by galectin-3 and galectin-1 inhibition with TD139, and it was fibroblast cell specific with no effect in epithelial cells.

## Galectin-3 promotes TGFβ activity

Galectin-3 stimulation in TMLCs and HLFs increased canonical TGFβ signaling. In the TMLCs the TGFβ activity increase was recombinant galectin-3 concentration

dependent. In HLFs the recombinant galectin-3 induced Smad2 phosphorylation was blocked by inhibiting the TGFβ receptor and by inhibiting galectin-3 and 1 activity with TD139. As recombinant galectin-3 stimulation had no effect on pSmad2 levels in epithelial cells, it suggests that galectin-3 does not directly activate the TGFβ receptor because stimulation of epithelial cells with TGFβ was able to induce Smad2 phosphorylation in epithelial cells. How galectin-3 may promote TGFβ signaling in a fibroblast specific mechanism will be explored in the next chapter.

CD98 is a transmembrane heterodimer protein comprised of SCL3A2 (4F2 heavy chain, solute carrier family 3 member 2) and SLC7A5 (4F2 light chain, solute carrier family 7 member 5). It regulates β-integrin signaling, and has key roles in cell survival, proliferation, adhesion and inflammation. CD98 has been shown to be a receptor for galectin-3 (Dong and Hughes, 1997). CD98 associates with integrin β1, where its crosslinking with the integrin leads to integrin mediated focal adhesion and PI3K activation (Rintoul *et al.*, 2002; Henderson *et al.*, 2004). In macrophages, galectin-3 binds to and crosslinks CD98 and via the association with integrin β1 CD98 stimulates PI3K activation to drive alternative macrophage activation (Alison C MacKinnon *et al.*, 2008). The importance of CD98 in disease was highlighted through the inhibition of both galectin-3 and CD98 in A549 epithelial cells leading to the decrease in TGFβ induced Smad2 phosphorylation (A C MacKinnon *et al.*, 2008). The decrease in TGFβ mediated pSmad2 was also confirmed through siRNA-mediated knockdown of galectin-3 and CD98 (A C MacKinnon *et al.*, 2008). My results show that CD98 is expressed by iHBECs and not by HLFs. I hypothesis that any recombinant

galectin-3 mediated Smad2 phosphorylation increases and TGFβ signaling decreases through galectin-3 inhibition is not through CD98. In epithelial cells, it is possible that the addition of recombinant galectin-3 to the cells leads to an increase in galectin-3-CD98 binding and therefore an inadequate galectin-3-TGFβ interaction to see any effect on pSmad2 levels via western blot.

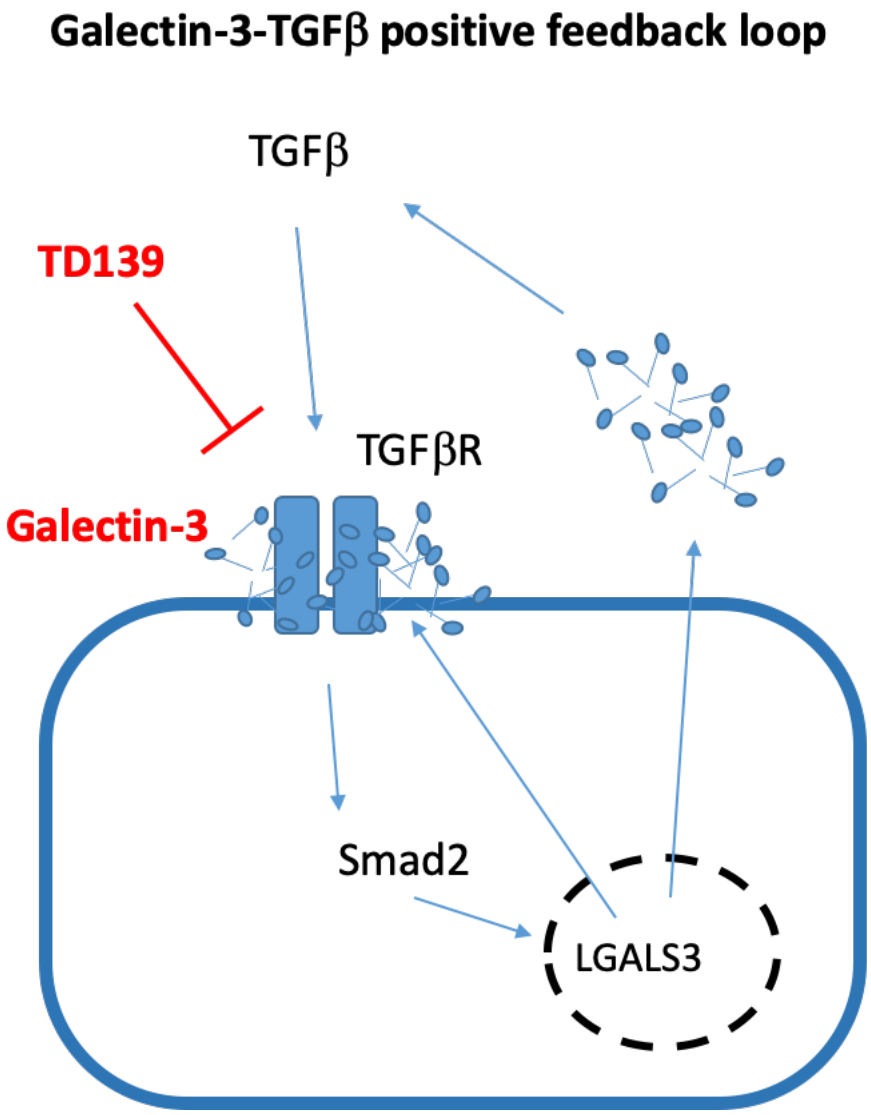



**Figure 6**. Figure illustrates the galectin-3-TGFβ feedback loop. TGFβ signaling can be blocked by inhibiting the action of galectin-3. TGFβ signaling promotes galectin-3 expression (data shown in

# Summary, limitations and future work

In summary, I have shown that galectin-3 inhibition in mouse fibroblasts leads to a decrease in canonical TGFβ signaling. In human fibroblasts however, inhibition of galectin-3, and possibly galectin-1, via TD139 led to the inhibition in Smad2 phosphorylation. TD139 however, had no effect on canonical TGFβ signaling is epithelial cells. Additionally, recombinant galectin-3 increased TGFβ signaling in fibroblasts but not in epithelial cells. This is thought to be through either a novel, unknown fibroblast specific receptor for Galectin-3 or the possibility that CD98 acts as a decoy receptor for Galectin-3 in epithelium. This data leads to the hypothesis of a galectin-3- TGFβ feedback loop (figure 4.6). Where galectin-3 inhibition attenuates TGFβ signaling, galectin-3 expression is increased in response to TGFβ stimulation (data shown in last chapter) and galectin-3 stimulation promotes TGFβ activity.

A limitation of my study is the use of immortalized epithelial cells to investigate the role of galectin-3 in epithelial TGFβ signaling. To become immortalized the cells would have to undergone significant mutations which could alter the biology of the cell and therefore do not completely mimic primary cells (Ramirez *et al.*, 2004). Therefore, to offer improved translatable data primary epithelial cell lines such as human bronchial epithelial cell (HBECs) should be used.

Another limitation of the study is within the effect of galectin-3 inhibition on TGFβ signaling study. It has been noted that galectin-3 levels vary between cell lines

and galectin-3 expression and levels can increase the longer the cells are grown (unpublished data from Mackinnon lab). Thus, in the study galectin-3 levels should have been measured and standardized to offer better comparisons between HLFs extracted from three different control patients, from three different IPF patients, and iHBECs.

Future work should investigate the role of galectin-1 on TGFβ signaling in fibroblasts and epithelial cells. Furthermore, to investigate the importance of galectin-1 and galectin-3 in the TGFβ signaling pathway, galectin-1 knockout cells could be treated with TD139 and stimulated with TGFβ. These studies will help distinguish whether galectin-1 or galectin-3 is mediating the effect of TD139.

Future work will also investigate the role of CD98 in galectin-3-TGFβ signaling in epithelial cells. As is it possible that galectin-3 has higher affinity for CD98 than TGFβ receptors in epithelial cells, use of a CD98 inhibitor cyanropicrin, to block the galectin-3-CD98 interaction whilst stimulate the epithelial cells with recombinant galectin-3 will help address whether CD98 is acting as a decoy receptor.

# Chapter 5: Mechanism of Galectin-3 induced TGFβ activation and signalling

# Chapter 5- Mechanism of Galectin-3 induced TGFβ activation and signalling

## Introduction

TGFβ plays a critical role in the development and progression of fibrosis, where it promotes numerous fibrotic pathways. When synthesised, TGFβ is released and stored as an inactive complex. Thus, to exert its biological effects, TGFβ needs to be activated.

Extracellular TGFβ concentrations are regulated, and its activity is regulated temporally and spatially. TGFβ is firstly synthesised as a propeptide with a mass of 75Dka. The propeptide is cleaved from the mature TGFβ in the trans Golgi. The cleaved propeptide known as the latency associated peptide (LAP) has a high affinity for TGFβ and non-covalently associates with the active TGFβ molecule forming the small latent complex (Annes, 2003). In turn, this latent complex, via a disulphide bond, binds to a latent TGFβ binding protein (LTBP) forming the large latent complex (Annes, 2003). When secreted, the LTBP attach to the ECM or cell surfaces where they are stored. The LTBPs thus determine the cellular and tissue localisation and distribution of TGFβ (Annes, 2003). As part of the large latent complex, TGFβ cannot bind to its receptors to exert its biological effects as the LAP acts as an inhibitor.

Therefore, TGFβ needs to be disassociated from this latent complex which is referred to TGFβ activation.

TGFβ can be activated through numerous different mechanisms such as through heat, oxidative reaction species, pH, thrombin, plasmin, MMPs, and by integrins (Sato and Rifkin, 1989; Taipale, Koli and Keski-Oja, 1992; Yu and Stamenkovic, 2000; Karsdal *et al.*, 2002; Jenkins, 2008). Integrins are heterodimeric transmembrane proteins which are capable to bidirectional signalling. They are composed of an $\alpha$ and β subunit, where the mammalian genome encodes 18 different $\alpha$ subunits and 8 different β subunits creating 24 different $\alpha\beta$ combinations. Integrins act as bidirectional cell receptors receiving and transmitting signals from outside and in the plasma membrane. This is known as inside-out and outside-in signalling (Wipff and Hinz, 2008). They are able to bind to ECM proteins such as collagen, laminin and fibronectin. Additionally, they are also able to bind to growth factors and cell surface ligands to mediate cell adhesion. Integrin activity is regulated by controlling the cytoskeletal organisation. Cytoskeletal reorganisation and integrin activation occurs through the activation of Ras homologous GTPases (Rho GTPases) (DeMali, 2003)

The αV family of integrins are able to bind to ligands through the arginine-glycine-aspartic acid (RGD) sequence. An RGD sequence is found in the LAP of TGFβ1 and TGFβ3, allowing their activation through αV integrins (Tatler and Jenkins, 2012).

At the moment αVβ6 mediated TGFβ activation is the best characterised mechanism for TGFβ activation. At the RGD motif, integrin αVβ6 bind to the LAP region, leading to conformational changes in the latent TGFβ complex, its dissociation, and consequential activation (Annes, 2003). As the activated TGFβ is not released into the extra cellular spaces it can only interact with TGFβ receptors on adjacent cells. Integrin αVβ6 is primarily expressed by lung epithelial cells (Saini, Porte, Weinreb, Violette, Wallace, McKeever, *et al.*, 2015). Injury related pathways such as thrombin and lysophosphatidic acid (LPA) signalling through the proteases-associated receptor 1 (PAR1) and LPA receptor 2 (LPAR2) have been associated with αVβ6 mediated TGFβ activation (Jenkins *et al.*, 2006). Activation of the receptors leads to RhoA and rho kinase, leading to cytoskeletal reorganisation, epithelial contraction, and a conformational change resulting in integrin αVβ6 binding to the latent complex and activating TGFβ (Jenkins *et al.*, 2006).

Mechanical stretch of lungs slices, epithelial cells, and fibroblasts have been shown to lead to the release of active TGFβ which is released from the latent complex (Froese *et al.*, 2016; John *et al.*, 2016; Bellaye *et al.*, 2017). The precise mechanism of this is yet unclear, however it is thought that the G protein coupled receptors are implicated in this (John *et al.*, 2016). The role of galectin-3 in mechanical stretch mediated TGFβ activation and signalling will be explored.

Galectin-3 has been shown in previous studies to interact with integrins. Galectin-3- integrin $\alpha 2\beta 1$, $\beta 1$, $\beta 3$, and $\alpha v\beta 3$ association has been shown to be crucial

in cell migration, adhesion, and angiogenesis (Friedrichs, Manninen, Daniel J. Muller, *et al.*, 2008; Lei *et al.*, 2009; Markowska, Liu and Panjwani, 2010; Priglinger *et al.*, 2013; Yang *et al.*, 2017).

## Aims and Objectives

As shown in the last chapter, galectin-3 promotes TGFβ activity in human lung fibroblasts but not in epithelial cells. The mechanism by which TGFβ signalling is increased by galectin-3 is unknown. Additionally, integrins involved in TGFβ activation in fibroblasts has still not been clearly defined. It is hypothesised that galectin-3 promotes TGFβ activity and signalling by increasing TGFβ activation through integrin mediated TGFβ activation and binding to the latent TGFβ complex. Thus, the aims of this chapter are:

1. To investigate the role of galectin-3 in stretch-induced TGFβ signalling in human lung fibroblasts.
2. To investigate the role of galectin-3 in LPA mediated TGFβ activation.
3. To investigate the role of galectin-3 in integrin mediated TGFβ activation.
4. To explore whether galectin-3 directly activates TGFβ by binding to the TGFβ-latent complex.

# Methods

## Cyclic cell stretch

Healthy and IPF HLFs were plated onto 6 well Bioflex plates which contain a flexible silicone membrane. Cyclical stretch is created by the Flexcell tension system (FX5000T) whereby a vacuum is created in the gasket on which the Bioflex plates are seated therefore deforming the flexible membrane on which the cells are adhered to by pulling the membrane downwards and creating equiaxial stretch. Cells are approximately 80% confluent at the start of the experiment. Once 80% confluent, cells are serum starved overnight. After serum starvation, media is removed, and treatments added: 50µM Alk5 inhibitor and 1µM TD139 to the relevant wells. The 6 well plates are then installed onto the gasket of the Flexcell tension system. To create a normal physiological environment, a stretch frequency of 20 cycles per minute (0.33Hz) was applied and elongation strain of 15%, which is equivalent to parameters seen in people.

After 48 hours of cell treatment and continuous cyclic cellular stretch, cells were lysed on ice and cell lysate were stored at -80$^{o}$C to await analysis. Cells were then scraped and transferred to microcentrifuge tubes. The samples were then centrifuged at 14,000 x g for 10mins at 4$^{o}$C. The supernatants were transferred to

microcentrifuge tubes and the pellets were discarded. The protein concentration was then determined using the Pierce BCA assay. The effect of galectin-3 inhibition on TGFβ activation and signalling in cell stretch was analysed by western blot by measuring psmad2 levels.

## MTT assay

After experimental cell treatment, cell supernatant was removed, and cells washed with PBS. Cells were incubated with MTT in medium for 10mins at $37^0$C. The MTT solution was then discarded and plates were allowed to dry overnight at room temperature. The cells containing the purple formazan were solubilised by the addition of DMSO. The absorbance was then read at 550nm using a spectrophotometer.

## Specific assays

Healthy and IPF HLFs were grown until ~80% confluent and DMEM was changed to serum free DMEM. After 24 hours of serum starvation the cells were pre-treated with β1 inhibitor (0.1-100nM), αvβ5 inhibitor (10µg/ml), galectin-3 inhibitors (1µM TD139, GB1211, GB1107, GB0149), αv inhibitor (10nM-1µM), or Alk5 inhibitor (50µM). Following 20mins of inhibitor pre-treatment cells were stimulated with 2ng/ml TGFβ (2 hours), 50µM LPA (4 hours), 50µM SFLLRN (PAR1 agonist) (4 hours),

or 10µg/ml recombinant human galectin-3 (2 hours). Following treatment, cells were lysed, scrapped, and stored at -80$^{o}$C to await analysis. The concentration of the cell lysates was determined via BCA assay. TGFβ activity was investigated by measuring the levels of psmad2 via western blot.

## Western blot

Proteins were separated via SDS-PAGE. 15-25µg of protein was loaded per lane in a 10% SDS-polyacrylamide gel. After electrophoresis stage of the western blot, protein was transferred from the gel to a polyvinylidene difluoride membrane (PVDF). After the transfer stage, the PVDF membranes were blocked with 5% milk (5% non-fat milk in TBS plus 0.1% Tween 20) for 1 hour at room temperature. The membranes were then incubated overnight at 4$^{o}$C with anti-Psmad2 primary antibody (Phospho-Smad2 (Ser465/467) (138D4) Rabbit mAb #3108, Cell Signalling Technology) at 1:1000 dilution in blocking buffer. Following overnight primary antibody incubation membranes were washed with TBST and then incubated with HRP-conjugated anti-rabbit secondary antibody (1:3000 dilution in blocking buffer) for 1 hour at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL.

Following visualisation of Psmad2, membranes were probed further for anti-Glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) (1:10000 dilution in blocking buffer) for 1-hour room temperature. Membranes were washed in TBST and

then be incubated with HRP-conjugated anti-rabbit secondary antibody (1:3000 dilution in blocking buffer) for 30mins at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL. GAPDH was used as the loading control.

Following visualisation of GAPDH, membranes were stripped using stripping buffer for 5 minutes. Membranes were washed in TBST and blocked with 5% milk (5% non-fat milk in TBS plus 0.1% Tween 20) for 1 hour at room temperature. The membranes were then incubated overnight at 4$^{o}$C with anti-smad2/3 primary antibody (smad2/3 Rabbit polyclonal Ab #3102, Cell Signalling Technology) at 1:1000 dilution in blocking buffer. Following overnight primary antibody incubation membranes were washed with TBST and then incubated with HRP-conjugated anti-rabbit secondary antibody (1:3000 dilution in blocking buffer) for 1 hour at room temperature. Blots were visualised using enhanced chemiluminescence (ECL) and exposure to Hyperfilm-ECL.

The western blot bands were quantified using ImageJ software. Results are expressed as a band density ratio of pSmad2 to Smad2, which indicates the relative expression of pSmad2.

## Solid-phase binding assay

High binding 96-well plates were coated with 2µg/ml recombinant TGFβ, or recombinant LAP, or recombinant LTBP1, or collagen, or PBS. The plates were incubated overnight to allow binding of the recombinant protein to the plate. Plates were then washed with PBS and blocked with 1% Bovine Serum Albumin for one hour. Plates were washed again with PBS and increasing concentrations of recombinant human galectin-3 (0, 0.01, 0.1, 1, 10µg/ml) was added the individual wells and incubated at room temperature for 4 hours. Wells were then washed with PBS and 100µl conjugated galetin-3 antibody was added to each well and incubated for 2 hours at room temperature. Wells were again washed and 100µl substrate solution was added and incubated for 30mins. 50µl stop solution was added and optical density was read using a spectrophotometer at 450nm with correction at 570nm.

## Co-immunoprecipitation

Co-immunoprecipitation was prepared according to the Universal Magnetic Co-IP Kit (Active Motif) protocol and reagents. Healthy HLFs were treated with 50µM of LPA for 4 hours. After treatment, lysed and concentration determined via BCA assay, 100µg of protein in addition of 500µl wash buffer and primary antibody (1mg/ml IgG, or 1:30 dilution galectin-3 primary antibody, αv integrin primary antibody 1:40 dilution) was incubated on a rotator for 2 hours at 4$^{o}$C. The samples were centrifuged at 11,000 x g for 30 s and supernatants were collected. 25µl protein G magnetic beads were added to each sample and samples were incubated for one

hour at 4°C on a rotator. Samples were then centrifuged for 30 seconds at 11,000 x g and the supernatant collected. The samples were then placed onto a magnetic stand and supernatant removed and the pellet resuspended in 20µl 4x LDS sample buffer containing 2.5% β-mercaptoethanol. The samples were then analysed by western blot where levels of galectin-3 and αv integrin was investigated.

## Co-culture reporter assay

MEFβ6 cells were seeded in a 96 well plate at a density of $5x10^4$ cells/ml. TMLCs were seeded with the MEFβ6 in direct co-culture, and also seeded alone at a density of $5x10^4$ cells/ml to measure the total active TGFβ. The galectin-3 Inhibitors was added at a concentration dependent manner (1nM-1000nM). Recombinant human TGFβ1 was added to relevant wells in a concentration dependent manner (62.5pg/ml-5000pg/ml). TGFβ1 treatments was used as a standard curve. After 16h incubation at 37°C, cells were washed in cold PBS and lysed with 50µl of reporter lysis buffer (Promega). The lysates were then transferred to a luminometry plate, and 100µl of luciferase reagent was added. Subsequently, the luciferase activity was immediately measured using FLOUstar omega multi-mode microplate reader (BMG Labtech, Ortenburg, Germany). All experiments were performed in triplicate and were repeated at least three times.

# Results

## Role of galectin-3 in stretch mediated TGFβ activation in fibroblasts

HLF from normal and IPF patients were seeded on BioFlex culture plates and stretched for 48hours on the Flexcell Tension System. Unstretched normal or diseased HLFs had no increase in pSmad2 levels (figure 5.1). However, 48 hours of cyclic stretch increased levels of pSmad2 in both control and disease cells (figure 5.1A and 5.1B). The increase in pSmad2 levels is completely blocked by inhibiting the TGFβ receptor with the Alk5 inhibitor (figure 5.1A and 5.1B) however galectin-3 inhibition did not significantly change the levels of pSmad2 (figure 5.1A and 5.1B).

As cell stretch increases TGFβ activity, I want to explore whether this was due to changes in galectin-3 expression. The expression change in galectin-3 in normal and IPF HLFs via qPCR had shown that cyclic stretch has no effect on galectin-3 expression in fibroblasts (figure 5.1C).

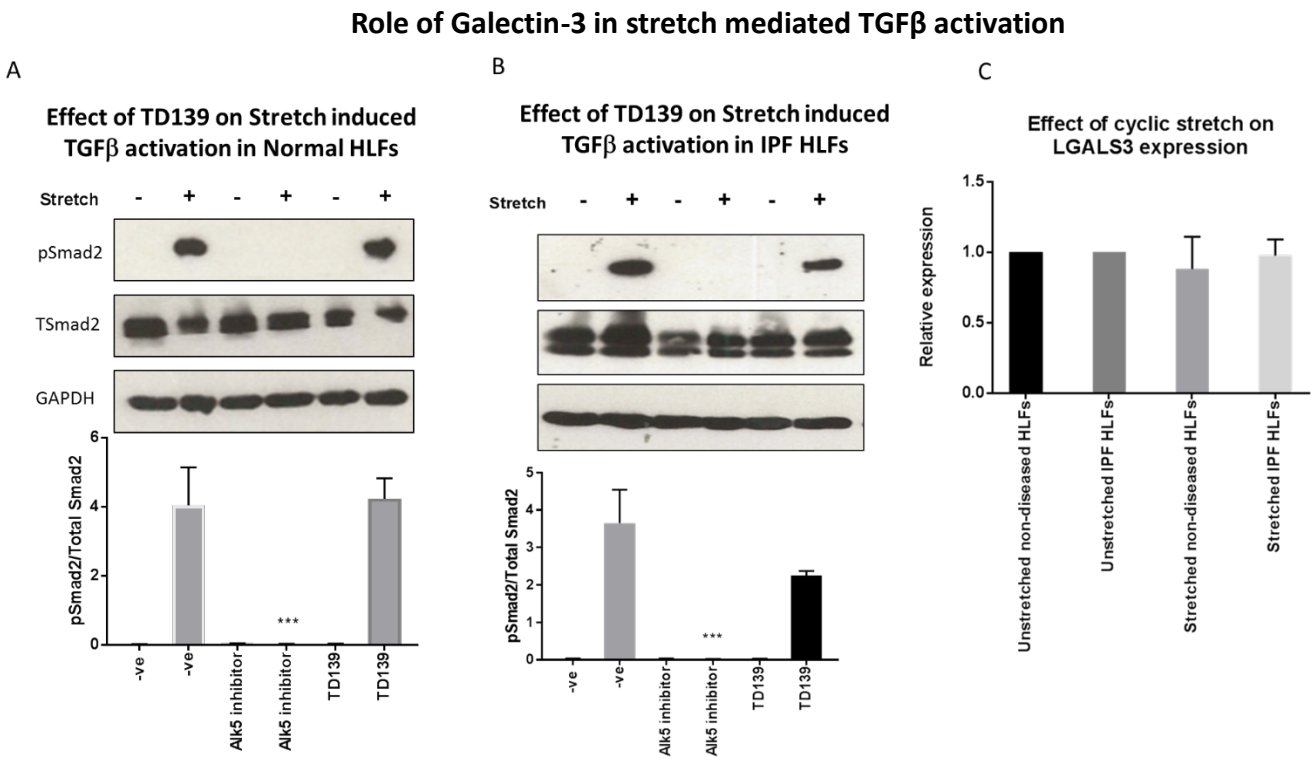



*Figure 5. 1*

The role of galectin-3 in stretch mediated TGFβ activation and signalling in healthy and IPF HLFs was investigated. HLF were stretched on a Flexcell tension system for 48hrs at 15% elongation and at a frequency of 0.3Hz. Expression of galectin-3 in response to stretch was investigated by qPCR (C). Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. *** $p<0.001$ as compared to cells treated with only TGFβ (+ve). N=3.

## Integrins involved in latent TGFβ activation

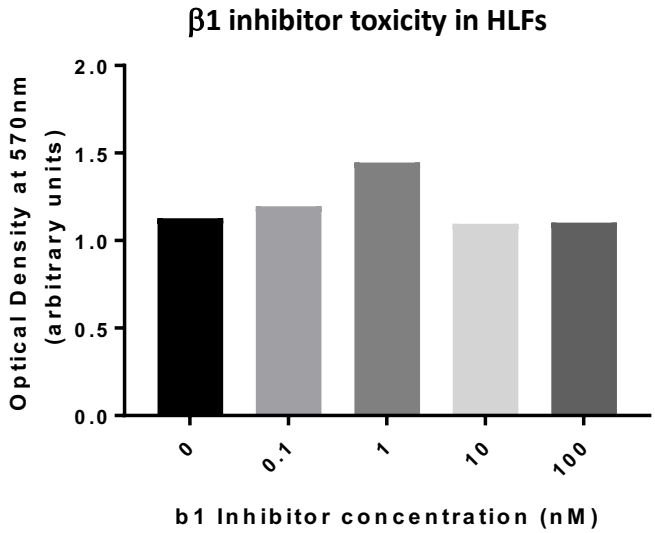



*Figure 5. 2*

Toxicity of integrin β1 inhibitor in HLFs was investigated via MTT assay. Effect of the inhibitor was investigated using increasing concentrations. N=1

Integrins have been shown to activate TGFβ by binding to the latent complex leading to the disassociation of the active TGFβ molecule from the latent complex. Fibroblast specific integrins that contribute to TGFβ activation was investigated. Additionally, β1 integrins have been shown to be involved in galectin-3 mediated response. Utilising a β1 small molecule inhibitor, I examined whether the inhibitors had any toxic effects on HLFs at a range of different concentrations (0.1-100nM) via an MTT assay (figure 5.2). Data shows that the β1 inhibitor between 0.1-100nM had no toxic effects on HLF cell viability. Thus, the β1 inhibitor was utilised for the study.

Stimulation of fibroblasts with LPA lead to increased pSmad2 increases (figure 5.3A and 5.3B). This increase in pSmad2 was blocked with increasing concentrations of the integrin β1 inhibitor to a similar level as the Alk5 inhibitor (positive control) at

concentrations of 10nM and 100nM (figure 5.3A). The IC50 for the β1 inhibitor is seen to be approximately 5.5nM.

αvβ5 has been shown to activate TGFβ thus the role of integrin αvβ5 was also investigated in TGFβ activation in HLFs. HLFs were stimulated with LPA in the presence of an anti-αvβ5 antibody (ALULA) and compared with an isotype control and the ALK5 inhibitor was used as a positive control. As shown in Fig 5.3B the anti-αvβ5 antibody blocked pSmad2 to a level observed with the Alk5 inhibitor, whereas the isotype control had no effect.

**Integrins involved in LPA/PAR1 mediated TGFβ activation in fibroblasts**

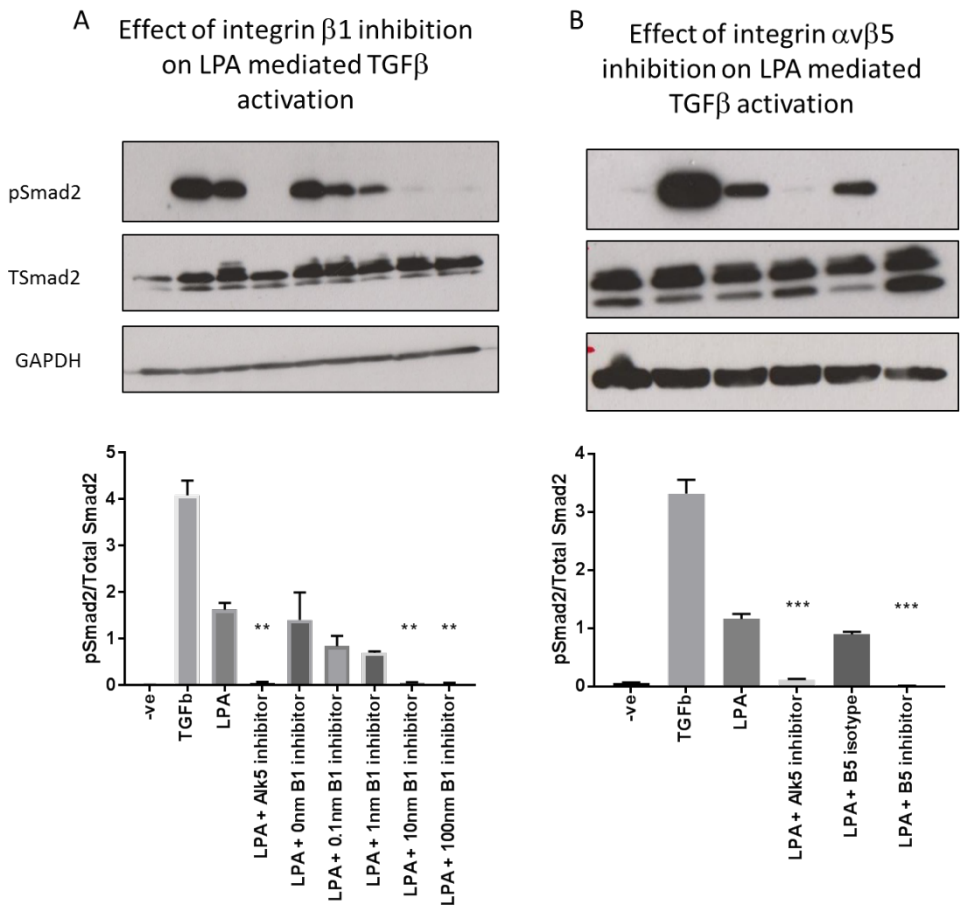



*Figure 5. 3*

The role of integrins in LPA mediated TGFβ activation was investigated. A, B, LPA mediated TGFβ activation can be blocked by inhibiting integrin β1 and αvβ5. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. * p<0.05, ** p<0.01, *** p<0.001 as compared to cells treated with only TGFβ (+ve). N=3.

## Role of galectin-3 in LPA mediated TGFβ signalling

To investigate whether galectin-3 was involved in GPCR agonist induced TGFβ activation HLFs were stimulated with LPA (figure 5.4A and 5.4B) or SFLLRN (PAR1 receptor agonist (figure 5.4C) in the presence of the galectin-3 inhibitors TD139, GB1211, GB1107 and TGFβ activity was measured by western blot and quantified by densitomtry. Stimulation with LPA and SFLLRN both led to increase in pSmad2 levels (figure 5.4). All three galectin-3 inhibitors reduced levels of LPA or SFLLRN mediated pSmad2 increase in HLFs to levels observed with an Alk5 inhibitor used as a positive control (figure 5.4). The data indicates that galectin-3 plays a role within the LPA/PAR1- TGFβ activation pathway.

**Galectin-3 Inhibition blocks LPA/PAR1-integrin mediated TGFβ activation in fibroblasts**

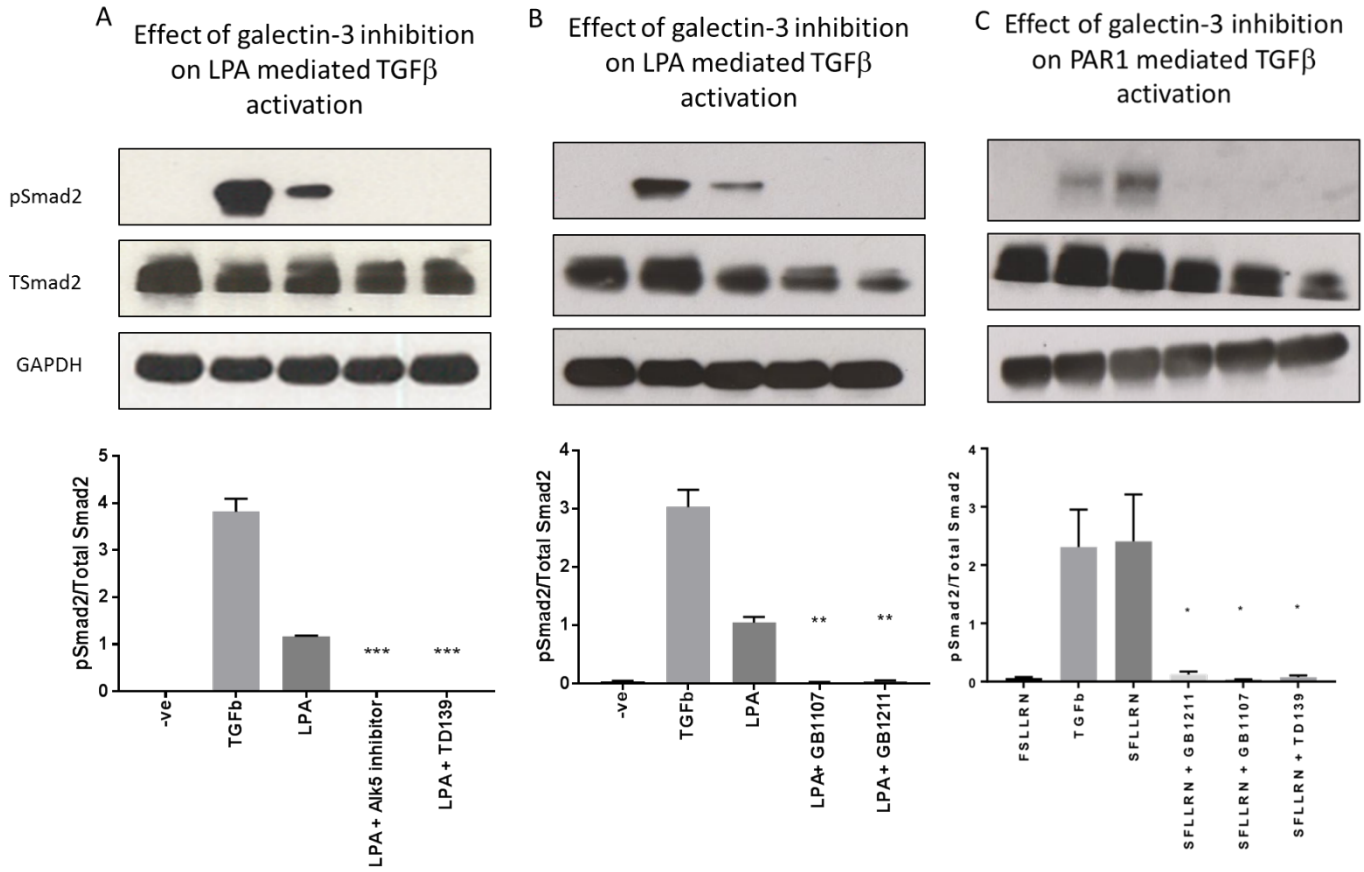



*Figure 5. 4*

The role of galectin-3 in LPA mediated TGFβ activation was investigated. (A, B) HLFs were stimulated with LPA and galectin-3 activity was blocked. C, HLFs were treated with the PAR1 agonist SFLLRN and galectin-3 activity was blocked. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ as compared to cells treated with only TGFβ (+ve). N=3.

As I have previously described galectin-3 is a ubiquitous glycoprotein, which can be expressed both within the cell and within the extracellular spaces. To delineate whether it is intracellular or extracellular galectin-3 that is involved in the LPA- TGFβ activation pathway I used an extracellular galectin-3 inhibitor (GB0149) which cannot pass the cell membrane. HLFs were preincubated with GB0149 and

then treated with 50µM of LPA for 4 hours and psmad2 was measured by western blot and the results quantified by deniostometry. LPA stimulation increased pSmad2 levels which was inhibited by GB0149 in a concentration dependent manner (figure 5.5). The IC50 for GB0149 is seen to be approximately 1µM.

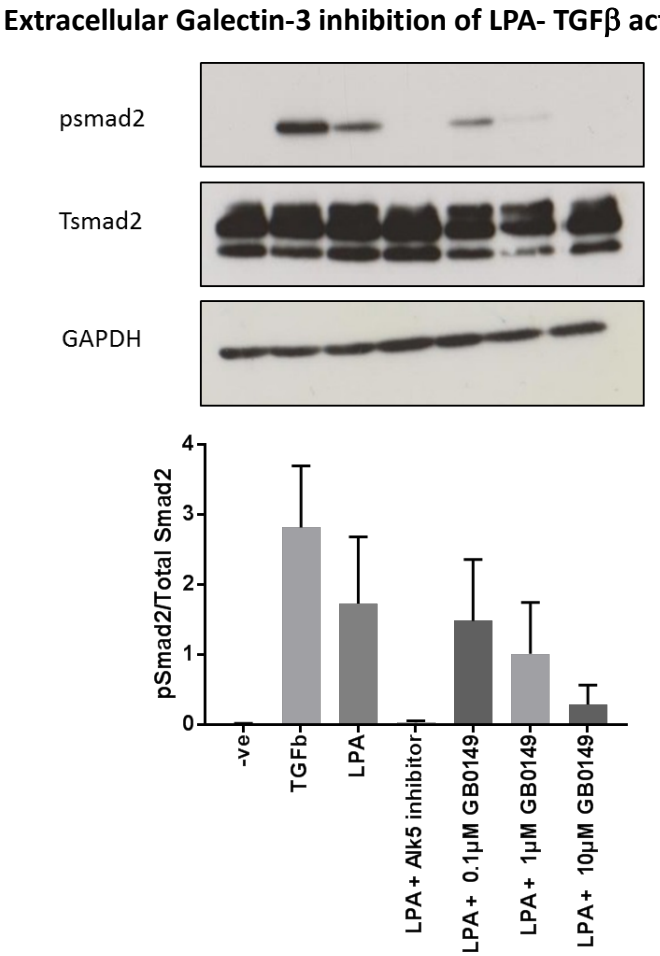



*Figure 5. 5*

The role of extracellular galectin-3 in LPA mediated TGFβ activation was investigated. HLFs were stimulated with LPA and extracellular galectin-3 activity was blocked. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. N=3.

## Role of galectin-3 in integrin mediated TGFβ activation

As results from figure 5.3 indicate LPA mediated TGFβ activation involves integrins β1 and αvβ5, and the data from figure 5.4 and 5.5 indicates galectin-3 is also involved in LPA induced TGFβ activation, the effect of integrin inhibition on recombinant galectin-3 signalling was investigated. HLFs were stimulated with recombinant galectin-3 and integrin β1 subunit, αvβ5, and αv subunit were inhibited using β1 (figure 5.6A), αvβ5 (figure 5.6B), and αv (figure 5.7) inhibitors. Recombinant galectin-3 stimulation increased pSmad2 levels (figure 5.6 and 5.7). Inhibition of the β1 integrin subunit did not inhibit galectin-3 mediated psmad2 increases at concentrations between 0.1nM-10nM (figure 5.6A). At 100nm β1 significantly decreased Psmad2. However, this is above the inhibitors selectivity and a failure to see a concentration dependent effect this decrease is most likely to be attributed to a non-selective effect. Inhibition of the integrin αvβ5 (figure 5.6B) and the αv subunit also did not inhibit galectin-3 induced pSmad2 levels.

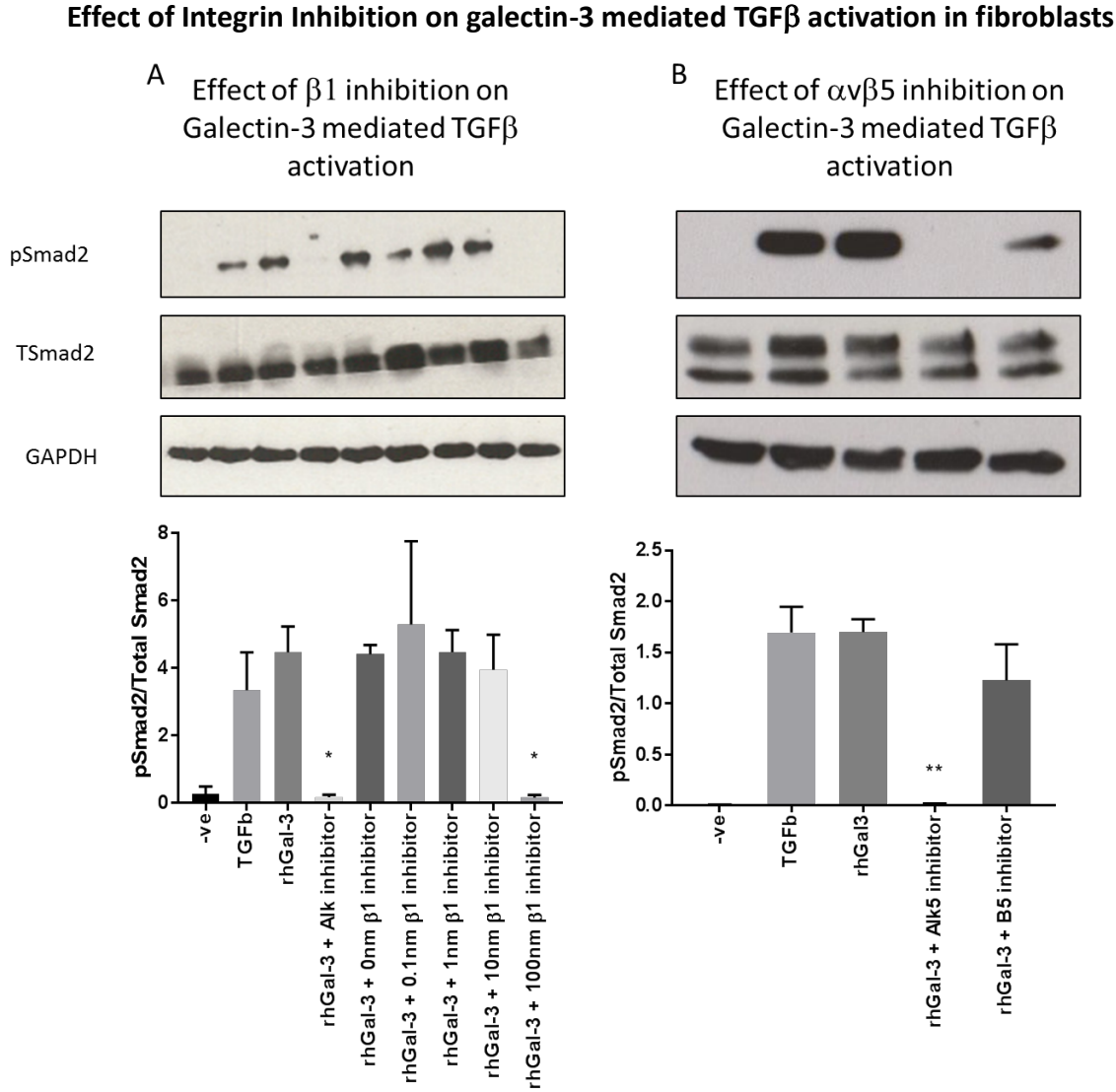



*Figure 5. 6*

The effect of integrin β1 (A) and αvβ5 inhibition (B) on galectin-3 mediated TGFβ activation and signalling was investigated. HLFs were stimulated with recombinant galectin-3 and integrins β1 (A) and αvβ5 (B) were inhibited. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. * $p<0.05$, ** $p<0.01$ as compared to cells treated with only TGFβ (+ve). N=3.

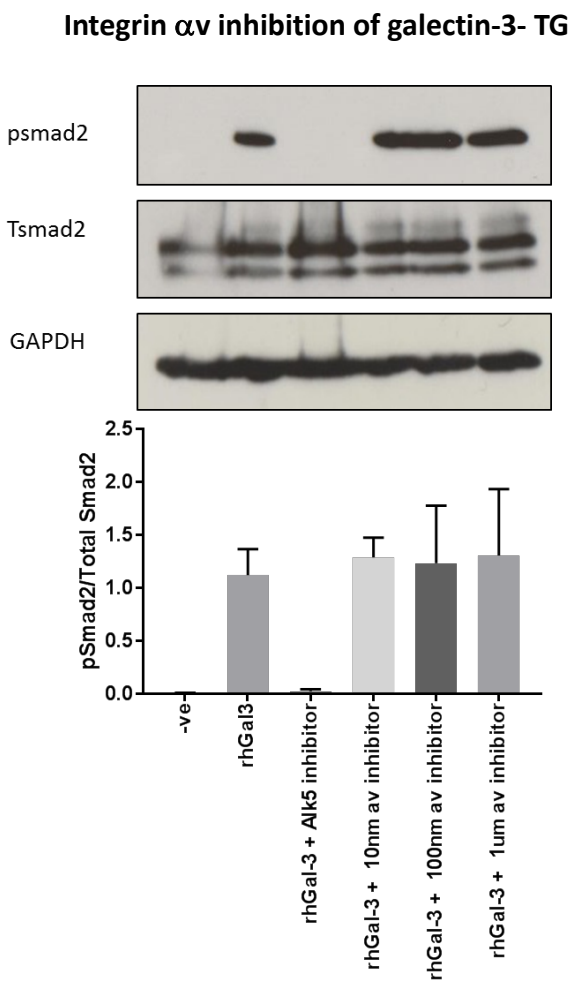



*Figure 5. 7*

The effect of integrin αv subunit inhibition on galectin-3 mediated TGFβ activation and signalling was investigated. HLFs were stimulated with recombinant galectin-3 and integrin αv subunit was inhibited. Western blot bands were quantified using densitometry. Quantity of Psmad2 was normalised to quantity of total smad2. N=3.

## Galectin-3 binding to the latent TGFβ complex

LPA induced pSmad2 was blocked by both integrin and galectin-3 inhibition. In contrast recombinant galectin-3 induced pSmad2 was not blocked by inhibiting integrins. One possibility to explain this apparent paradox is that galectin-3 is

involved in TGFβ activation distal to the integrin mediated effects on the small latent complex. Therefore, I investigated whether galectin-3 might facilitate integrin mediated TGFβ activation via effects on the large latent TGFβ complex. I used a solid-phase binding assay, where 96 well plates were coated with PBS (negative control), collagen (positive control), recombinant LAP, and recombinant LTBP1. Increasing concentrations of recombinant galectin-3 were added and after HRP conjugated anti-galectin-3 antibody incubation the optical density was measured. Galectin-3 binding to collagen was significantly increased at 0.1-10μg/ml (figure 5.8B) compared with PBS control. There was no increase in optical density in the addition of galectin-3 to the TGFβ (figure 5.8A) and LAP (figure 5.8B) coated wells indicating galectin-3 does not directly bind to active TGFβ and LAP. In the wells coated with LTBP1, optical density at 450nm was significantly increased at the addition of 10μg/ml galectin-3 (figure 5.8C). This therefore indicates that galectin-3 binds to the LTBP1 region of the latent TGFβ complex.

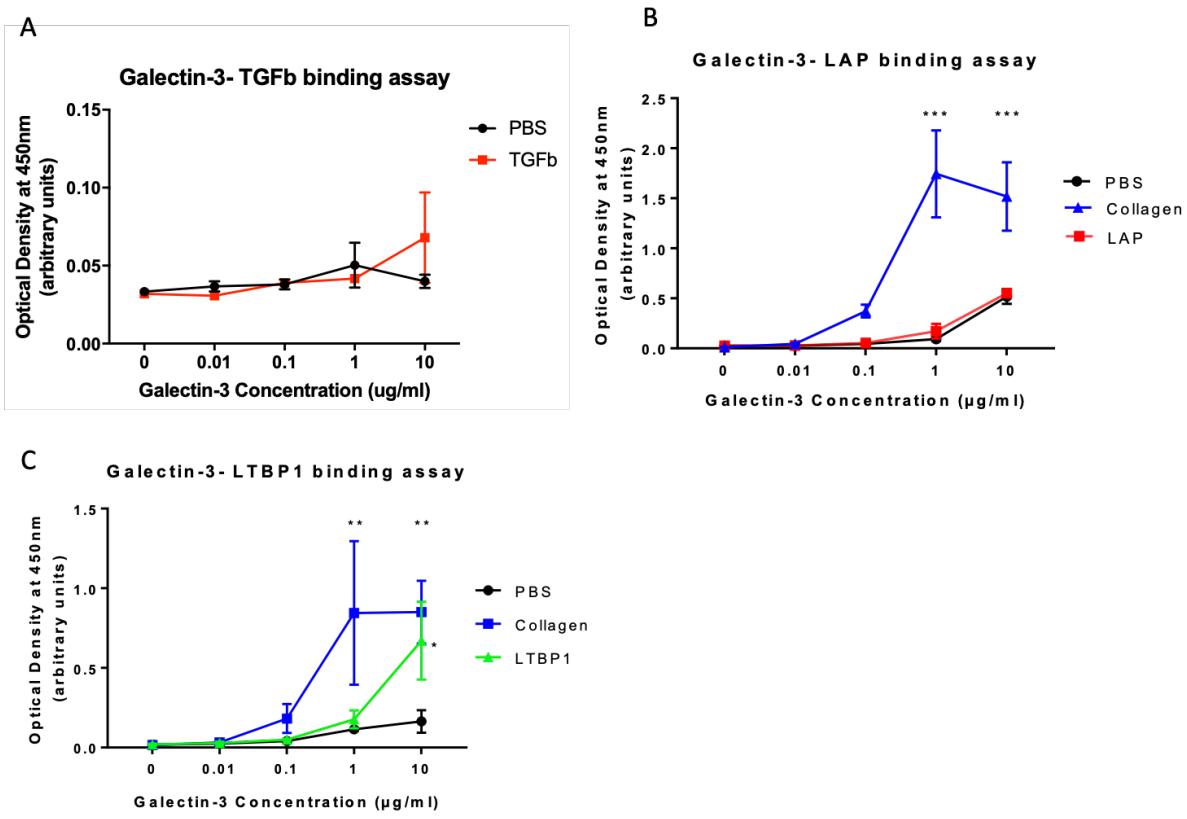



*Figure 5. 8*

The effect of galectin-3 binding to the latent TGFβ complex was investigated by a binding assay. 96 well plates were coated overnight with 5µg/ml collagen (used as positive control), (A) 2µg/ml TGFβ (B) 2µg/ml LAP, (C) 2µg/ml LTBP1, or PBS (negative control). Increasing concentrations of galectin-3 was then added followed by a galectin-3 conjugated anti-body. Substrate solution was then added, and optical density was measured at 450nm and corrected at 570nm. * $p<0.05$, *** $p<0.001$. All experiments were performed in triplicates and repeated at least 3 times- N=3/4.

## Galectin-3-αv integrin subunit interaction

Because galectin-3 directly binds to the LTBP1 region of the large latent TGFβ complex, and prior studies have indicated galectin-3 directly interact with integrins I investigated galectin-3 and αv integrin subunit interactions by co-immunoprecipitation to determine if the galectin-3 facilitated integrin mediated TGFβ activation through co-association with LTBP1 and the αv subunit.

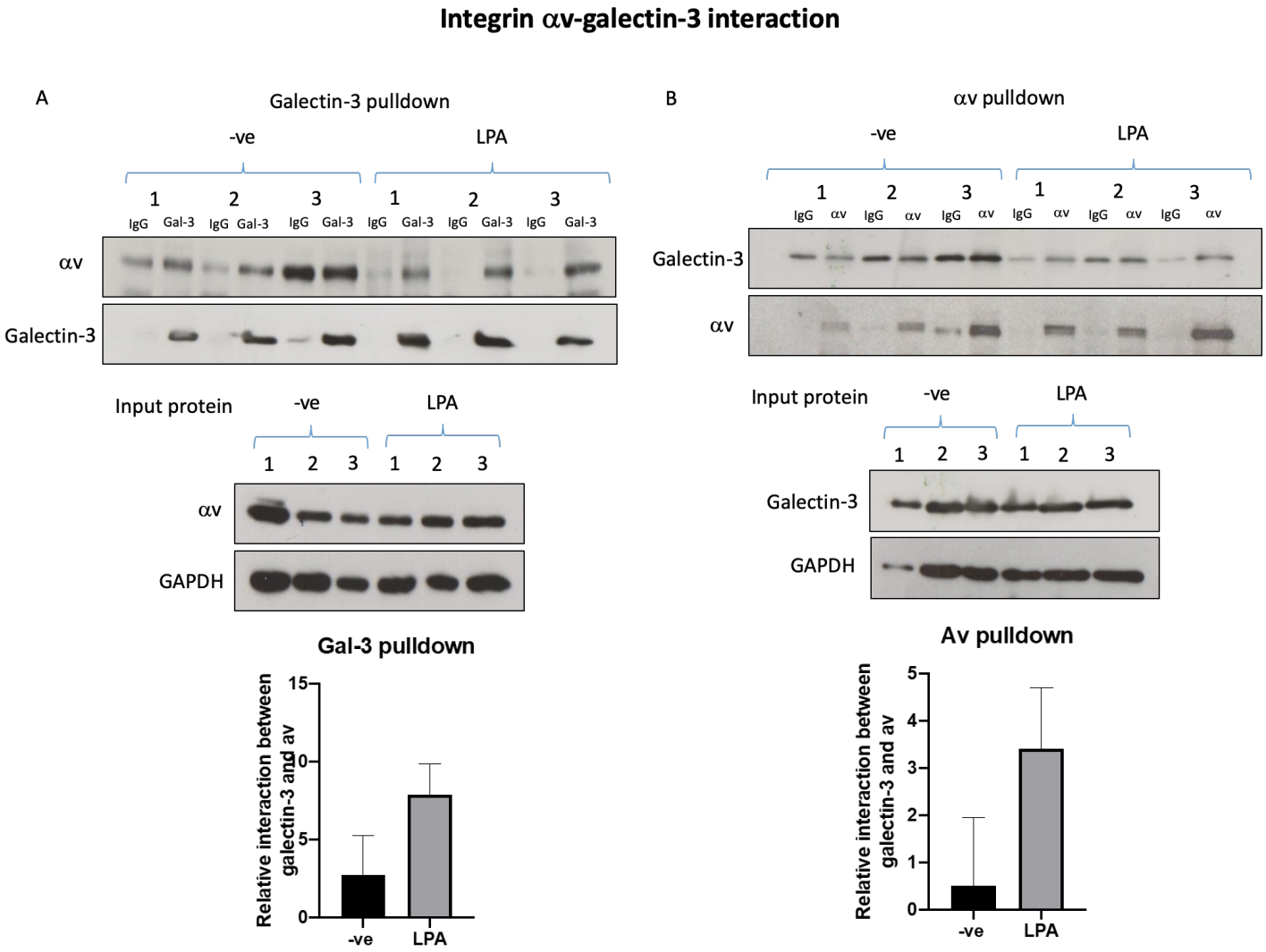



*Figure 5. 9*

The interaction between galectin-3 and αv integrin subunit was investigated via co-immunoprecipitation. HLFs were treated with LPA for 4 hours. 100µg of protein was used for the immunoprecipitation. (A) Galectin-3 was immunoprecipitated and αv was detected via western blot. 10µg of protein was used as an input control. (B) αv was immunoprecipitated and galectin-3 was detected via western blot. 10µg of protein was used as an input control. Relative interaction between galectin-3 and integrin av was calculated via densitometry. N=3.

LPA increased TGFβ activation through integrins β1 and αvβ5 (figure 5.3), which can be inhibited by blocking action of extracellular galectin-3 (figure 5.5). This highlights that galectin-3 is important in LPA-TGFβ activation where it could bind to integrins to lead to TGFβ activation in this pathway. HLFs were treated with LPA or control for 4 hours and 100µg of protein was immunoprecipitated- galectin-3 immunoprecipitation (figure 5.9A) and αv immunoprecipitation (figure 5.9B) followed by αv (figure 5.9A) and galectin-3 (figure 5.9B) western blots. In the unstimulated control HLFs galectin-3 interaction with the αv subunit varies according to HLFs donor cell lines. However, Figure 5.9A shows that when stimulated with LPA and galectin-3 is immunoprecipitated, αv levels increase indicating an interaction between galectin-3 and αv integrin subunit after LPA stimulation. When HLFs were stimulated with LPA and αv integrin was immunoprecipitated, galectin-3 levels increased compared to the IgG control (figure 5.9B). This supports an interaction between galectin-3 and αv integrin upon LPA stimulation.

# Discussion

## Summary of findings

The work in this chapter investigated the mechanism by which galectin-3 promotes TGFβ activity in fibroblasts. I demonstrated that galectin-3 does not play a role in cyclic stretch-mediated TGFβ activation.

The activation of LPA receptor 2 via LPA leads to integrin αvβ5 and β1 mediated TGFβ activation. The inhibition of galectin-3 in this pathway (via GB1211, GB1107 and TD139) blocks TGFβ activation and signalling. Blocking the action of extracellular galectin-3 (via GB0149) also blocks LPA mediated integrin-TGFβ activation and signalling. Furthermore, LPA stimulation led to the association of galectin-3 to the αv integrin subunit. Highlighting that galectin-3 binds to the αv integrin subunit promoting TGFβ activation during LPA receptor 2 activation.

Furthermore, as shown in the last chapter recombinant galectin-3 promotes TGFβ signalling. Recombinant galectin-3 mediated TGFβ signalling could not be blocked by pharmacologically inhibiting the integrins αvβ5, β1 and αv. Subsequently, I have shown that galectin-3 cannot bind to the active TGFβ molecule and the LAP region of the latent TGFβ complex. However, galectin-3 binds to the LTBP1 region of the large latent TGFβ complex. Thus, it is thought that galectin-3 binds to the LTBP1

region of the large latent TGFβ complex to promote the release and activation of TGFβ and lead to increased TGFβ signalling.

The chapter therefore demonstrates two different mechanism by which galectin-3 promotes TGFβ signalling. It is also worth considering a third possible mechanism which utilises the two mechanisms described above. In this mechanism, extracellular galectin-3 binds directly to both the LTBP1 region of the large latent TGFβ complex and to the αv integrin to form a lattice structure and consequently promoting αv binding to the latent TGFβ complex at the RGD motif. This leads to the release and activation of TGFβ and therefore an increase in TGFβ signalling.

## Role of galectin-3 in stretch-mediated TGFβ activation

Cyclic stretch models in studies have therefore been produced to mimic normal inspiratory and expiratory breathing cycles and tension seen in the lungs. I mimicked normal breathing in my cyclic stretch model by stretching fibroblasts at 15% elongation and at a frequency of 0.3Hz.

I had previously shown in this thesis that TGFβ signalling can be inhibited by blocking galectin-3 action (chapter 4). Thus, I investigated whether galectin-3 inhibition affects stretch mediated TGFβ signalling. Cyclic stretch is a known mediator for TGFβ activation (Hamzeh, Sridhara and Alexander, 2015; Froese *et al.*, 2016; John *et al.*, 2016; Shimbori *et al.*, 2019). Cyclic stretching of human renal proximal

epithelial cells (HK2 cells) lead to the increase in TGFβ1 release (Hamzeh, Sridhara and Alexander, 2015). Hamzeh *et al*. (2015) had shown that stretching HK2 cells leads to the increase in β1 integrin expression. Knocking down β1 integrin via siRNA in the cells resulted in the decrease in TGFβ1 expression induced by stretch (Hamzeh, Sridhara and Alexander, 2015). Thus, showing that the integrin β1 is vital in the activation of TGFβ1 during cyclic stretch.

Furthermore, John *et al.* 2016 had shown that G-protein couple receptors are crucial to stretch-mediated TGFβ activation. Knock-down of the G-protein $G_{q/11}$ in mice reduced stretch mediated TGFβ activation by epithelial cells. Accordingly, fibrotic lungs strips exposed to mechanical stretch were shown to produce increased TGFβ signalling and pSmad2 levels (Froese *et al.*, 2016). The levels of TGFβ activation increased with increasing duration of stretch (5-15mins) and increasing magnitude (5-20mN) (Froese *et al.*, 2016). Interestingly, the levels of stretch induced TGFβ activation was higher in fibrotic lungs compared to normal lungs (Froese *et al.*, 2016). This is attributed to the increased stiffness of the fibrotic lung tissue. Soft ECM does not provide sufficient elastic resistance to the large latent TGFβ complex from the traction force generated between the cell surface integrin and LAP. However, in more ridged ECM enough traction force is generated to allow the active TGFβ molecule to be released from the LAP domain. And further studies delineated that the pathway involved in the stretch-mediated TGFβ activation involved Rho kinase and integrin αv (Froese *et al.*, 2016).

My observation that pharmacologically inhibiting galectin-3 in my cyclic stretch model had no effect on stretch mediated TGFβ activation and pSmad2 increases would suggest that galectin-3 is not involved in CMS induced TGFβ activation pathway. The expression of galectin-3 was also unchanged in stretched fibroblasts.

## Role of Galectin-3 in Integrin mediated TGFβ activation

My results show LPA stimulation and PAR1 activation leads to TGFβ activation and increases in pSmad2 level in fibroblasts. This is consistent with known effects of TGFβ activation in epithelial cells (Xu *et al.*, 2009) and fibroblasts (Tager *et al.*, 2008) and ASMCs (Tatler *et al.*, 2011). TGFβ is released by cells in an inactivate latent complex, and thus needs to be activated to exert is biological effect. The latent TGFβ complex is composed of the active TGFβ molecule bound to LAP. This smaller complex then is bound to a LTBP domain forming the large latent TGFβ complex. When the latent TGFβ complex is released it localises onto the ECM where is it stored. In lungs of patients with IPF, LTBP1 is significantly increased, where expression was significantly increased within the fibroblastic foci (Leppäranta *et al.*, 2012).

LPA and thrombin are G-protein coupled receptor agonists and are released from platelets following injury. They have been shown to induced $\alpha v\beta 6$ mediated TGFβ activation and implicated in the development of IPF (Howell, Laurent and Chambers, 2002; Tager *et al.*, 2008). To induce $\alpha v\beta 6$ mediated TGFβ activation, LPA

and thrombin signalling through the G-protein couple receptors LPAR2 and PAR1 induces cytoskeletal changes via the downstream activation of RhoA and Rho kinase (Xu *et al.*, 2009). The cytoskeletal change allows for the αvβ6 integrin to bind to the RGD sequence on the LAP region of the latent TGFβ, and subsequently the release of the active TGFβ molecule. This increase in LPA induced pSmad2 was inhibited by blocking integrins and galectin-3. Although recombinant galectin-3 stimulation in fibroblasts induced TGFβ activation as measured by pSmad, the inhibition of integrins αvβ5, β1 and αv had no effect on recombinant galectin-3 induced TGFβ activation. This indicated that galectin-3 is sufficient but not necessary for TGFβ activation.

To understand the mechanism by which galectin-3 promotes TGFβ activation. I investigated whether galectin-3 can bind directly to extracellularly to the large latent TGFβ complex to promote TGFβ activation. The large latent TGFβ complex is composed of LTBP1, LAP and the active TGFβ molecule. For the active TGFβ molecule to be released from its latent complex it must separate from the LAP domain. The disassociation of the active TGFβ molecule from LAP can be achieved through numerous pathways. MMP2 and MMP9, known TGFβ activators, are able to cleave the latent complex at the LTBP domain resulting in the release of the large latent TGFβ complex from the matrix (Annes, 2003). Furthermore, MMPS are able to proteolytically cleave LAP allowing the release of the active TGFβ (Annes, 2003). The disassociation of the active TGFβ molecule from LAP can also occur through integrin binding via the RGD sequence.

Using solid-phase binding assays I show here that galectin-3 does not bind to the active TGFβ molecule or LAP at the concentrations of galectin-3 used in the study. However, galectin-3 binds to the LTBP1 domain of the large latent complex. LTBP1 anchors the latent TGFβ complex to the ECM and supports TGFβ activation by creating traction when integrins bind to LAP (Justin P. Annes *et al.*, 2004; Wipff *et al.*, 2007). This traction force generated between the cell surface integrin and ECM allows the release of the active TGFβ molecule from LAP (Justin P. Annes *et al.*, 2004; Wipff *et al.*, 2007). In stiffer ECM, as seen in fibrosis, larger traction forces are generated to allow the active TGFβ molecule to be released from the LAP domain. I therefore hypothesised that galectin-3 binding at the LTBP1 site of the large latent complex, forms a lattice structure between the latent TGFβ complex and the ECM thus tightly anchoring LTBP1 to the matrix. Thus, upon binding of the cell surface integrin to the tightly anchored latent TGFβ complex, enough traction force would be generated to release the active TGFβ molecule from the LAP domain.

The structure of galectin-3 is composed of a CRD region and an N-terminal domain. The CRD has high affinity for N-glycosylated proteins (N-acetyllactosamine). N-acetyllactosamine are reoccurring sequences found in branched N-glycans and the affinity of galectins increases with increasing branches. The N-terminal domain allows a galectin-3 monomer to form multimers via the association of other galectin-3 monomers. This allows galectin-3 to form crosslinks and cluster with integrins and cell surface receptors and form lattices consisting of both integrins and receptors. Studies have shown that the galectin-3 mediated cross-linking of receptors and

integrins mediate the activation of certain signalling pathways (Partridge, 2004; Lagana *et al.*, 2006). Galectin-3 mediated clustering has also been associated with endocytosis of β1 integrins (Furtak, Hatcher and Ochieng, 2001). Additionally, galectin-3 forms complexes with α3β1 and mediates the adhesion of α2β1 to collagen (Fukushi, Makagiansar and Stallcup, 2004; Friedrichs, Manninen, Daniel J Muller, *et al.*, 2008). MacKinnon *et al.* 2008 had shown that galectin-3 induces alternative macrophage activation by cross-linking integrin β1 and CD98 and forming a lattice. Additionally, another possible mechanism for galectin-3 induced TGFβ activation could be galectin-3 binding to αv integrins in fibroblasts and the LTBP1 domain of the TGFβ complex and consequently forming a lattice between the two. The formation of an integrin-latent TGFβ complex lattice via galectin-3 would allow the integrin to directly bind to the RGD sequence of the LAP domain resulting in the release of the active TGFβ molecule and increased TGFβ signalling.

The large latent complex of TGFβ is an extracellular complex that maintains TGFβ in its latent inactive state. Further support of the proposed hypothesis that galectin-3 acts to co-localise the αv subunit with LTBP1 to facilitate integrin mediated TGFβ activation is that the extracellular galectin-3 inhibitor was able to inhibit LPA induced TGFβ activity. Indicting that extracellular galectin-3 rather than intracellular effects LPA- TGFβ activity.

I show here that upon the activation of the LPAR2 receptor, galectin-3 associates with the integrin subunit αv. Galectin-3 binds to N-glycosylated proteins

which are found on the cell surface such as receptors and integrins, and in the ECM such as laminin and fibronectin (Woo *et al.*, 1990; Hughes, 2001; Kariya *et al.*, 2010). Galectin-3 and integrin interactions have been noted in numerous studies, where galectin-3 has been shown to associate with integrins $\alpha 2\beta 1$, $\beta 1$, $\beta 3$, and $\alpha v\beta 3$ (Friedrichs, Manninen, Daniel J. Muller, *et al.*, 2008; Lei *et al.*, 2009; Markowska, Liu and Panjwani, 2010; Priglinger *et al.*, 2013; Yang *et al.*, 2017). Galectin-3 has been shown to bind to $\alpha v\beta 3$ in angiogenesis (Markowska, Liu and Panjwani, 2010). Furthermore, the structure of the integrin $\alpha v$ revealed that the integrin is heavily glycosylated and consequently allowing for galectin-3 to bind. Galectin-3 promotes $\beta 1$ mediated cell adhesion to fibronectin and laminin (Margadant *et al.*, 2012). Intriguingly, integrin $\beta 1$ activation increased galectin-3 expression through the demethylation of the LGALS3 promoter (Margadant *et al.*, 2012).

Yang *et al*. 2017 demonstrated that galectin-3 binding at the integrin $\alpha v\beta 1$ formed lattices with other integrins, with the cluster size increasing in a concentration dependent manner. Interestingly, the increased integrin clustering resulted in increased integrin lateral motility (Yang *et al.*, 2017). Consequently, the formation of galectin-3-integrin lattices and increased integrin motility along the cell surface could enhance integrin-LAP binding. I therefore propose that upon the activation of the LPAR2 and PAR1 receptors, Rho A and Rho kinase activation and cytoskeletal reorganisation, galectin-3 binds to the extracellular domains of integrins $\alpha v\beta 1$ and $\alpha v\beta 5$ on fibroblasts. Galectin-3 binding at the integrins form lattice

structures with other integrins, enhancing integrin-LAP binding and induce TGFβ activation.

## Summary, limitations and future work

In summary I have shown that galectin-3 promotes TGFβ activity in fibroblasts and binds to both the latent TGFβ complex and to αv integrins. I hypothesise that galectin-3 can activate TGFβ via the following mechanisms (1) galectin-3 binding to αv integrins and forming a lattice complex with other integrins therefore enhancing integrin-LAP binding. (2) galectin-3 binding to the LTBP1 region of the large latent TGFβ complex and tightly anchoring the latent complex to the ECM resulting in little traction force needed for TGFβ to be released. (3) galectin-3 binding and forming lattices between αv integrins and the latent TGFβ complex, therefore increasing accessibility of the integrin to bind to LAP leading to TGFβ release.

A limitation of my study is that unlike utilising a monoclonal antibody targeting avb5 which is highly specific I used a small molecule inhibitor for targeting β1. Nevertheless, any off-target effect was negated by using the inhibitor in a concentration dependent manner (0.1nM-100nM). However, as seen in figure 6A, the inhibitor appeared to have an off-target effect at 100nM.

Another limitation of the study involves the galectin-3-αv co-immunoprecipitation experiment. Although in the galectin-3 pulldown experiment,

no bands were present in LPA stimulated fibroblasts IgG controls and convincingly showed an increase in αv in galectin-3 pulldown compared to IgG controls. However, in unstimulated fibroblasts and αv pulldown immunoprecipitation experiments bands were present in the IgG control lanes. This indicates that the co-immunoprecipitation experiment needs to be optimised through decreasing antibody concentrations or increasing wash steps. Further experiments will involve optimising the galectin-3-αv integrin co-immunoprecipitation experiment.

# Chapter 6: Discussion

# Chapter 6- Discussion

## Summary of important results from this thesis

IPF is a chronic and irreversible disease with a poor prognosis. The pathogenesis of the disease is poorly understood. However, the current hypothesis of IPF development and progression involves repeated injury to the lung epithelium in a genetically susceptible individual leading to the damage of the epithelial layer. This is followed by dysregulated wound repair responses involving fibroblasts migration to the site of injury, fibroblast activation, and fibroblast transformation to myofibroblasts. Additionally, epithelial cell injury leads to the loss of integrity to the alveolar epithelial membrane resulting in increased infiltration of inflammatory cells such as macrophages. Myofibroblasts continuously release ECM proteins such as collagen which deposit. The ineffective ECM removal leads to ECM accumulating leading to fibrosis and the progressive nature of the disease.

TGFβ is central to the IPF disease process where is has been shown to play roles in fibrotic pathways involved in epithelial cell apoptosis, ineffective epithelial cell migration, epithelial cell to EMT, fibroblast activation, fibroblast transformation to myofibroblast, synthesis of collagen by myofibroblasts (Hagimoto *et al.*, 2002; Kim *et al.*, 2006; Scotton and Chambers, 2007; Yu *et al.*, 2008). TGFβ plays a pivotal role in many biological processes, directly targeting TGFβ as a therapeutic target results in the risk of severe adverse effects. Thus, a target involved in TGFβ activation or

activity in specific cell types could minimise the risk of adverse effects seen in global TGFβ inhibition.

MacKinnon et al. 2012 had shown that galectin-3 -/- mice were protected from experimentally induced lung fibrosis. Furthermore, they had shown that galectin-3 -/- cells had reduced response to TGFβ. Thus, the hypothesis of the thesis was that galectin-3 is involved in TGFβ signalling and resulting in activation of fibrotic pathways leading to fibrosis. Inhibition of galectin-3 thus can block TGFβ signalling and be used for IPF treatment.

The work in this thesis has shown that galectin-3 promotes TGFβ activation through an integrin dependent manner, promoting TGFβ signaling. Furthermore, my work has shown galectin-3 expression is increased in the lungs of IPF patients compared to healthy individuals and in the lungs of mice treated with bleomycin compared to controls.

The thesis has demonstrated the important role galectin-3 plays within the TGFβ signaling pathway. MacKinnon *et al.* 2012 had shown that galectin-3 knock-out cells showed reduced responses to TGFβ stimulation. Furthermore, removal of galectin-3 resulted in the reduction in TGFβ receptor expression at the cell surface. Galectin-3 binds to the polylactosamine residues on the TGFβ receptor, therefore a loss of galectin-3 would result in a decrease in galectin-3 binding to the TGFβ receptors and therefore a decrease in TGFβ receptor retention (Partridge, 2004). My

data in this thesis had shown that blocking galectin-3 and 1 action blocked TGFβ signaling in fibroblasts but not in epithelial cells. This highlights that galectin-3 could be a desirable target for IPF treatment. Furthermore, TGFβ promoted galectin-3 expression in IPF fibroblasts. This observation could lead to the possibility of the existence of a galectin-3- TGFβ feedback loop in IPF, where TGFβ increases galectin-3 expression and in-turn galectin-3 promotes TGFβ activation and signaling (as summarized in figure 4.6).

Additionally, I have shown that recombinant galectin-3 promotes TGFβ signalling in fibroblasts but not in epithelial cells. It is hypothesized that the specific nature of galectin-3 action in HLFs compared to epithelial cells could be due to CD98 acting as a decoy receptor. Data presented in this thesis had shown that CD98 is expressed by iHBECs and not by HLFs. In epithelial cells, it is possible that the addition of recombinant galectin-3 to the cells leads to an increase in galectin-3-CD98 binding and therefore an inadequate galectin-3-TGFβ interaction to see any effect on pSmad2 levels via western blot. This will need to be investigated in further studies.

Integrins are able to activate TGFβ. The αV family of integrins are able to bind to ligands through the RGD sequence. An RGD sequence is found in the LAP of TGFβ1 and TGFβ3, allowing their activation through αV integrins (Tatler and Jenkins, 2012). Recombinant galectin-3 mediated TGFβ signalling could not be blocked by pharmacologically inhibiting the integrins $\alpha v\beta 5$, $\beta 1$ and $\alpha v$. Subsequently, I have shown that galectin-3 cannot bind to the active TGFβ molecule and the LAP region of

the latent TGFβ complex using the concentrations of galectin-3 tested. However, galectin-3 binds to the LTBP1 region of the large latent TGFβ complex. Thus, one mechanism of galectin-3 induced TGFβ action could be galectin-3 binding to the LTBP1 region of the large latent TGFβ. It is thought that galectin-3 clustering to this site tightly anchors the latent complex to the ECM resulting in little traction force needed for TGFβ to be released. This promotes the release and activation of TGFβ and leads to increased TGFβ signalling. This hypothesis is summarised in figure 6.1.

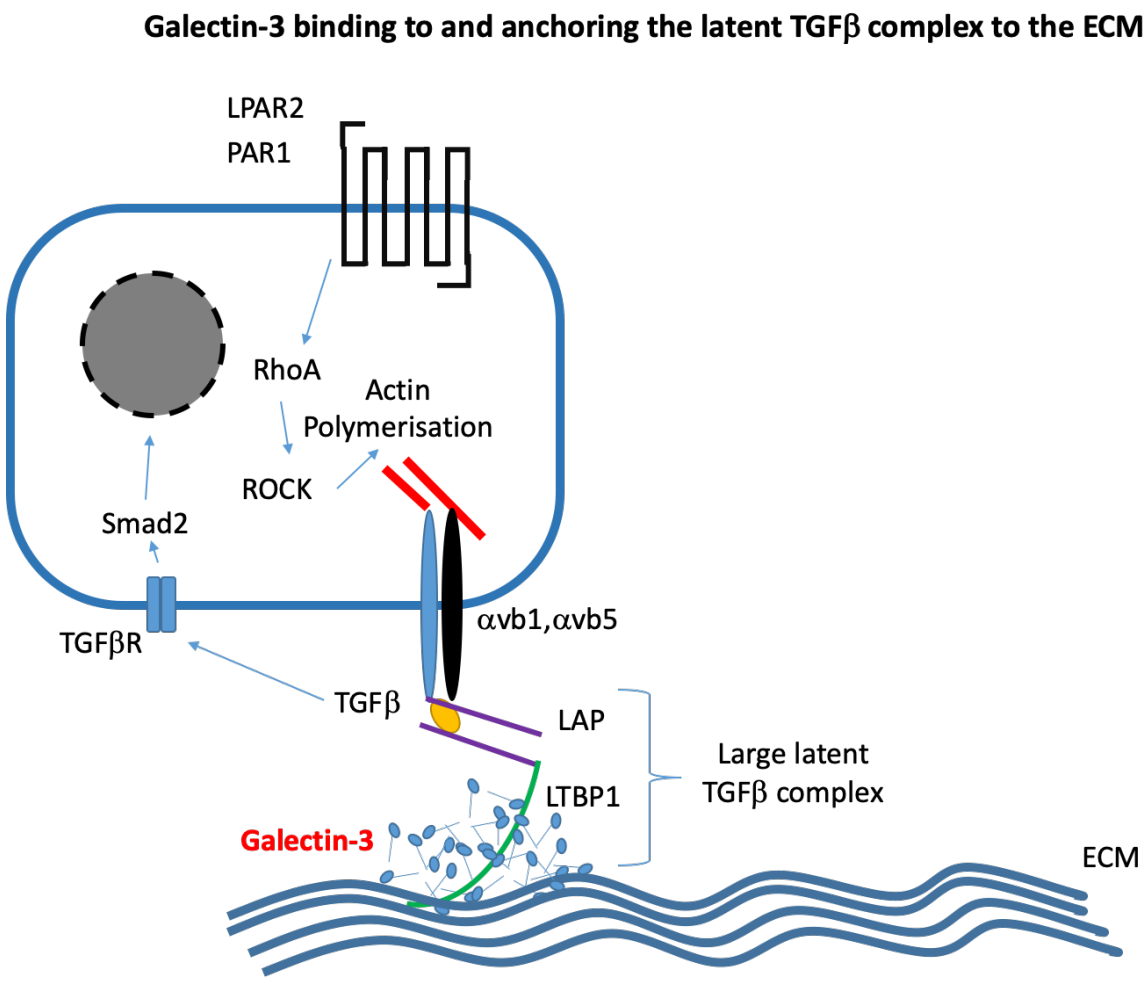



*Figure 6. 1*

Galectin-3 binds to the LTBP1 region of the large latent TGFβ complex and tightly anchoring the latent complex to the ECM resulting in little traction force needed for TGFβ to be released.

LPA and thrombin are G-protein coupled receptor agonists and are released from platelets following injury. Studies have shown activation of the receptors to lead to αvβ6 mediated TGFβ activation which is implicated in the development of IPF (Howell, Laurent and Chambers, 2002; Tager *et al.*, 2008). LPA and thrombin signalling through the G-protein couple receptors LPAR2 and PAR1 induces cytoskeletal changes via the downstream activation of RhoA and Rho kinase (Xu *et al.*, 2009). The cytoskeletal change allows for the αvβ6 integrin to bind to the RGD

sequence on the LAP region of the latent TGFβ, and subsequently the release of the active TGFβ molecule. My results show LPA stimulation and PAR1 activation leads to TGFβ activation and increases in pSmad2 level in fibroblasts. Pharmacological inhibition of integrins β1 and αvβ5 block LPA induced TGFβ activation. Highlighting, integrins β1 and αvβ5 induce LPA mediated TGFβ activation in fibroblasts. I showed that LPA and PAR1 mediated TGFβ activation is blocked by inhibiting the action of extracellular galectin-3. No effect was seen on the inhibition of galectin-3 on αvβ6 mediated TGFβ activation.

I therefore propose another possible mechanism where upon the activation of the LPAR2 and PAR1 receptors, RhoA and Rho kinase activation and cytoskeletal reorganisation, galectin-3 binds to the extracellular domains of integrins αvβ1 and αvβ5 on fibroblasts. Galectin-3 binding at the integrins form lattice structures with other integrins, enhancing integrin-LAP binding and induce TGFβ activation (summarised in figure 6.2). This observation was noted by Yang *et al*. 2017, where they demonstrated that galectin-3 binding to integrins formed lattices with other integrins.

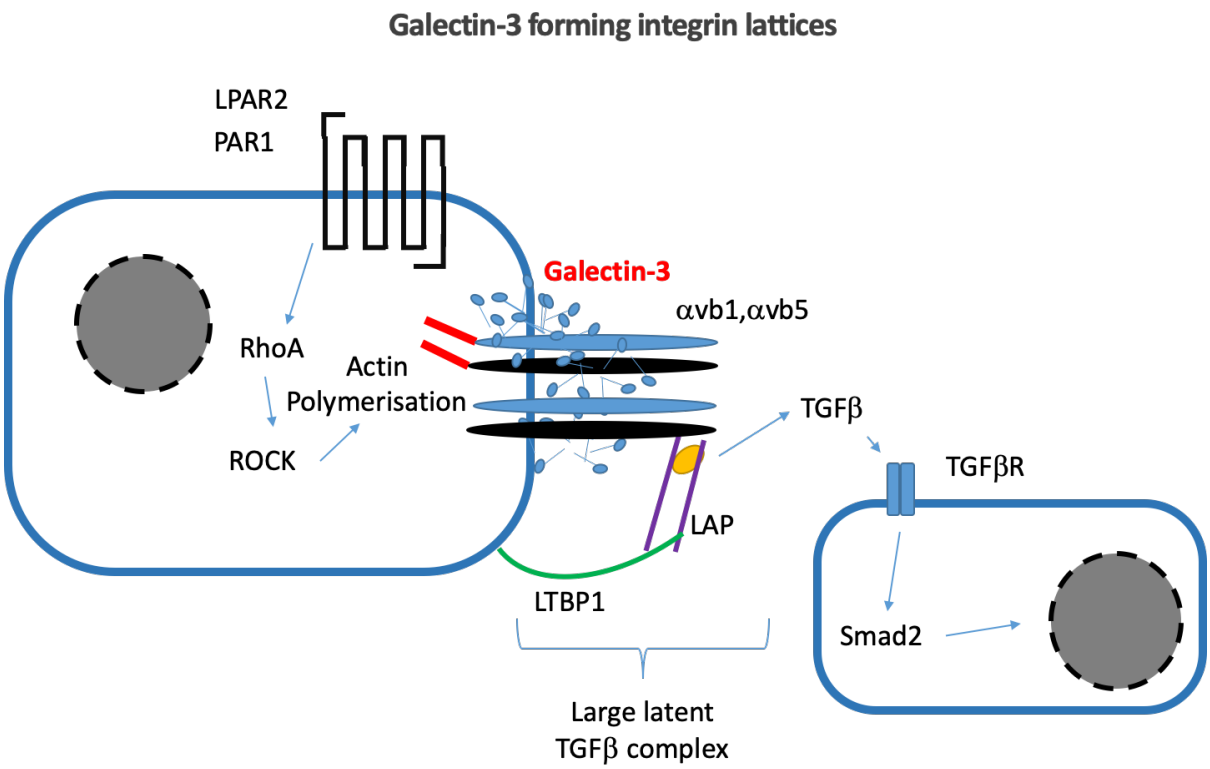



*Figure 6. 2*

Galectin-3 binding to αv integrins and forming a lattice complex with other integrins therefore enhancing integrin-LAP binding.

Additionally, another possible mechanism for galectin-3 induced TGFβ activation unites the two mechanisms described earlier. It is thought that galectin-3 binds to both αv integrins on fibroblasts and the LTBP1 domain of the TGFβ complex, and consequently forming a lattice between the two. The formation of an integrin-latent TGFβ complex lattice via galectin-3 would allow the integrin to directly bind to the RGD sequence of the LAP domain, resulting in the release of the active TGFβ molecule and increased TGFβ signalling. This is depicted in figure 6.3.

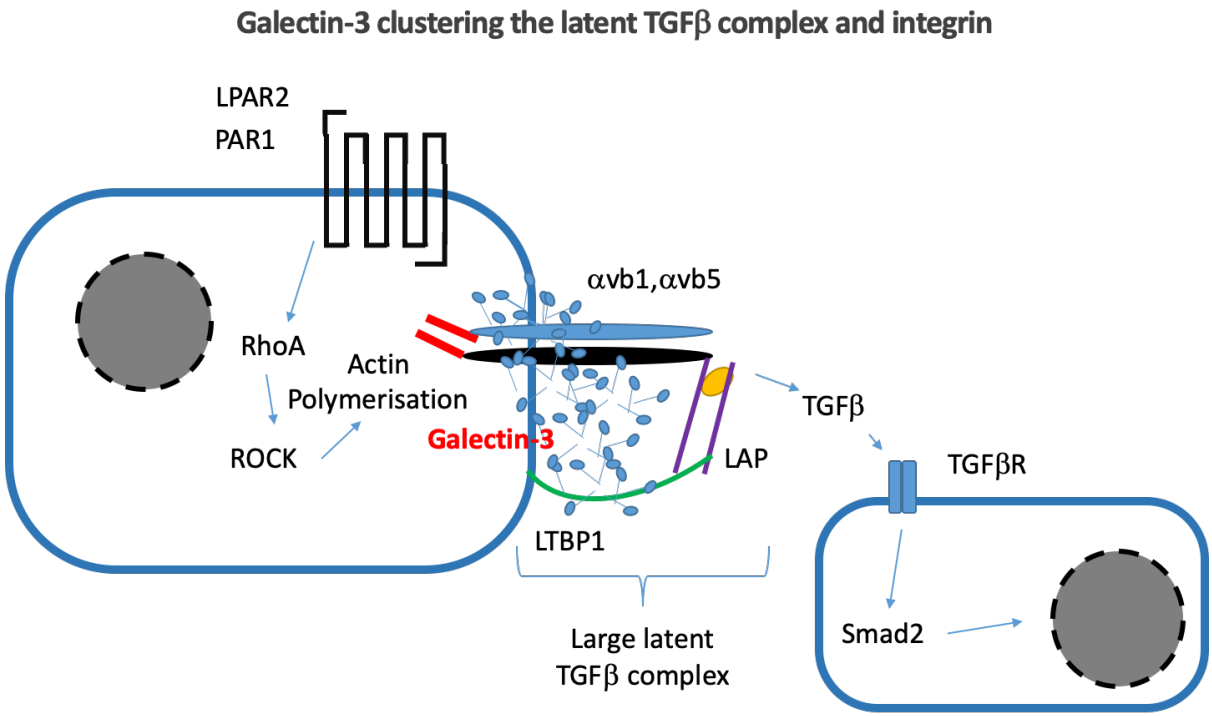



*Figure 6. 3*

Galectin-3 binding and forming lattices between αv integrins and the latent TGFβ complex, therefore increasing accessibility of the integrin to bind to LAP leading to TGFβ release.

## Future work

I showed that TGFβ stimulation promoted galectin-3 expression in IPF HLFs but not in healthy HLFs or IHBECs after 24 hours treatment. Future experiments would explore more timepoints in investigating TGFβ stimulation on galectin-3 mRNA expression and galectin-3 secretion. The work will also investigate the increased galectin-3 mRNA expression after TGFβ treatment demonstrated by IPF HLFs. The

work will look at the potential protective mechanism seen in the healthy HLFs and which could be dysregulated in IPF fibroblasts resulting in increased galectin-3 mRNA levels and expression which therefore can contribute to IPF disease development and progression.

TD139 is a galectin-3 and galectin-1 inhibitor. My data had shown that TD139 blocked TGFβ signaling in HLFs. Thus, future work should investigate the role of galectin-1 on TGFβ signaling in fibroblasts and epithelial cells. Furthermore, to investigate the importance of galectin-1 and galectin-3 in the TGFβ signaling pathway, galectin-1 knockout cells could be treated with TD139 and stimulated with TGFβ. These studies will help distinguish whether galectin-1 or galectin-3 is mediating the effect of TD139.

I had shown that CD98 is expressed by IHBECs but not HLFs. Future work will also investigate the role of CD98 in galectin-3-TGFβ signaling in epithelial cells. It is hypothesized that CD98 acts as a decoy receptor in epithelial cells, thus preventing the galectin-3 induced effects on TGFβ signaling seen in fibroblasts. As is it possible that galectin-3 has higher affinity for CD98 than TGFβ receptors in epithelial cells, use of a CD98 inhibitor cyanropicrin, to block the galectin-3-CD98 interaction whilst stimulate the epithelial cells with recombinant galectin-3 and measures TGFβ signaling will help address whether CD98 is acting as a decoy receptor.

Within my thesis I proposed 3 potential mechanisms by which galectin-3 promotes TGFβ activation. Thus, additional future work will further investigate galectin-3 binding and clustering to the integrin site and the latent TGFβ complex. I will attempt to examine where galectin-3 binds to on the integrin site, which β subunits (if any) galectin-3 binds to, and where galectin-3 binds to on the LTBP1 region of the latent TGFβ complex. I will also investigate whether galectin-3 is able to form a lattice to bridge multiple integrins together and/or with the latent TGFβ complex. This work with provide a conclusive mechanism by which galectin-3 promotes TGFβ activation and signalling.

## Overall conclusion

TGFβ signalling is central in fibrotic pathways seen in IPF. Because TGFβ plays vital role in many biological pathways its direct inhibition leads to severe adverse effects. The findings outlined in this thesis have provides evidence that TD139 blocks TGFβ signalling in fibroblasts but not in epithelial cells. Furthermore, the findings in the thesis have shown galectin-3 promotes TGFβ activation through potentially binding at the (1) LTBP1 region of the latent TGFβ complex, (2) integrin site, (3) both. The mechanisms still need further investigation, however if correct it will provide a better understanding of the pathogenesis and progressive nature of IPF.

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