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Research Article

Antifibrotic Drug Target Protein (RXFP1) is Expressed in Idiopathic Pulmonary Fibrosis Lungs

J Rai[1,2]*, W Mao[1,2], G Westall2, G Snell[2], CS Samuel[1] and SG Royce[1,2]

[1]*Department of Pharmacology, School of Biomedical Sciences, Monash University, Clayton, Victoria, Australia*

[2]*Department of Medicine, Central Clinical School, Monash University, Prahran, Victoria, Australia*

***Corresponding Author**: J Rai, Department of Pharmacology, School of Biomedical Sciences and Department of Medicine, Central Clinical School, Monash University, Prahran, Victoria, Australia.

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Abstract

Background and Aims: Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease of the lungs of unknown aetiology. Once diagnosed, it is usually fatal within 2.5 - 3.5 years. There is no effective treatment apart from lung transplantation and even then there is a 40% chance of rejection (chronic lung allograft dysfunction; CLAD). This too is characterized by fibrosis, especially around the airways in the transplanted lung. Due to its potent anti-fibrotic effects the relaxin/RXFP1 (relaxin receptor) system has been mooted as a possible therapeutic target for IPF. RXFP1 is expressed in normal mammalian lung but for a relaxin-based therapy to be effective it is important to ascertain whether RXFP1 protein is expressed in diseased IPF lung tissue.

Methods: Formalin-fixed paraffin-embedded unused donor (n = 10) and IPF lung tissue (n = 10) was obtained from the Alfred Lung Biobank and stained for RXFP1 (H-160, Santa Cruz) and fibrotic markers by immunohistochemistry. Staining was quantified with the positive pixel count algorithm, Leica Aperio Imagescope.

Results: RXFP1 protein was strongly expressed in both donor lung tissue and in that from the IPF patients. The receptor was located to most resident lung cell types especially in the airways and was also expressed in IPF usual interstitial pneumonia fibrotic lesions. There are no significant difference in expression between the two groups.

Conclusions: This is the first study to examine RXFP1 protein expression using this primary antibody with specificity validated by Western blot. The result will be further validated using a mass spectrometry approach that can be applied to RXFP1 measurement in bronchoalveolar lavage fluid. This study may have positive implications if relaxin-based therapies for IPF and CLAD are to be developed.

Keywords: *Antifibrotic; RXFP1; Idiopathic Pulmonary Fibrosis*

Introduction

Research Proposal

Ameliorating the profibrotic milieu in chronic lung pathology

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic disease of the lungs of unknown aetiology. Once diagnosed, it is usually fatal within 2.5 - 3.5 years. Currently available pharmacological treatments merely slow the disease progression. As such the only option for patients with IPF is lung transplantation. Apart from the obvious risks associated with surgery and the lack of donor lungs, there is a high chance of rejection of the allograft (40% at 5 years post-transplant, with a subsequent 50% 2-year mortality thereafter once initiated). Interestingly, regardless of pre-existing disease, the major feature of rejected lungs is peri-bronchial fibrosis (airway remodelling) and fibrosis occluding the small airways; also known as obliterative bronchiolitis (OB). As well as rejection these features are associated

with poor lung functional outcomes. Ideally what is required for IPF patients, particularly those that are transplant recipients, is a therapy that can safely reverse fibrosis in the lung in the parenchyma and especially around the airways.

The recombinant human form of the peptide hormone relaxin, serelaxin (RLN), has attracted intense recent interest for its anti-fibrotic and vasodilatory properties [1], culminating in RLN being awarded Breakthrough Designation (2013) by the FDA for the treatment of acute heart failure. Of note in the RELAX-AHF Phase III trial a single RLN infusion resulted in reduced mortality more the 120 days later [2]. After initial findings on the effect of RLN on pulmonary fibrosis [3], which are mediated through Relaxin Family Peptide Receptor 1 (RXFP1), CIA/CIB have more recently been developing it as a treatment for the airway remodelling and fibrosis associated with asthma [4-6].

Our intention with this application is to expedite clinical trials of RLN in lung transplant recipients where we anticipate that treatment is highly likely to reduce airway remodelling-associated rejection (given our well-established published findings). Simultaneously, we will conduct the pre-clinical evaluation of RLN in IPF cell culture and exacerbated-mouse models; as well as optimal delivery methods to the small airways - that is mandatory before any IPF trials can be commenced. Both the post-transplant and preclinical work detailed will advance our knowledge and bring us closer to using RLN as a treatment for IPF, transplant recipients and other lung diseases.

Hypothesis

The RLN/RXFP1 system can be used to treat the airway remodelling associated with IPF and post-transplant lung fibrosis.

To develop RLN as a suitable treatment for IPF and post-transplant lung fibrosis, we will:

Aim-1: A) Evaluate the effects of RLN administration to chronic bleomycin-induced PF, with LPS- epithelial damage stimulated exacerbations; and B) Demonstrate that RLN can be successfully nebulized, retain bioactivity and reach target tissues including the small airways. This will help to bridge the gap from experimental-to-clinical studies.

Aim-2: A) Characterize relaxin and RXFP1 expression in patients with IPF and transplant recipients; and B) Determine the effects of RLN treatment on markers of IPF-induced fibrosis using IPF patient and donor control lung fibroblasts *in vitro*.

Background and Preliminary Data

The problem: IPF is a fatal disease where functional lung parenchyma is replaced by non-functional scar tissue. As this progression occurs, accelerated by acute exacerbations, there is an irreversible decrease in oxygen diffusion capacity. There is a lack of pharmaceutical treatments for IPF, and available treatments remain essentially palliative, slowing disease progression at best. Drugs under development are either non-efficacious or are associated with several side-effects [7]. Effective ways of getting drugs into the lungs are also required to maximize delivery to the cells deep in the lung where the disease is initiated and promulgated. The only decisive treatment for IPF is lung transplantation. However, rejection after lung transplantation (including transplants in IPF, cystic fibrosis and chronic obstructive pulmonary disease (COPD)), known as chronic lung allograft rejection (CLAD), is a common occurrence and is characterized by peribronchial fibrosis (airway remodelling) as well as OB, where a fibrotic lesion grows in the small airways.

The opportunity: Reversal of remodelling/fibrosis and delivery all the way to the small airways and parenchyma. We have demonstrated that RLN therapy can reverse airway remodelling that also characterizes chronic allergic airways disease (asthma) [4-6] and can reverse scarring in solid organs [8]. We also have the technology to delivery RLN, retaining the bioactivity of the peptide all the way to the initiating and effector cells in these diseases (namely the epithelial cells and the myofibroblasts that stiffen the lung and secrete collagen).

RLN, an antifibrotic and organ-protective drug: The natural hormone RLN relaxes the uterus for childbirth, by regulating extracellular matrix composition. RLN was discovered in 1926 but interest has re-emerged in RLN because of its strong antifibrotic and vasodilatory properties. Recombinant human RLN was given Breakthrough Status by the FDA and in the RELAX-AHF (acute heart failure Phase III) trial; as a single infusion of RLN was able to greatly improve prognosis more than 120 d later [1,2] (see Figure to the left). There are many organ-protective functions of RLN (see Figure to the right, above); that are clinically relevant to treating lung diseases including IPF.

Our significant discoveries (relevant to this application)

- **The RLN-/- mouse** spontaneously develops an age-related pulmonary fibrosis phenotype, replacing normal lung tissue with fibrotic congestion [3]. This phenotype was be rescued by systemic administration of recombinant human (rh)RLN. Relaxin receptor (RXFP1; formerly LGR7) -/- mice also develop age-related pulmonary fibrosis, strongly demonstrating the pivotal role of the RLN/RXFP1 system in protecting from lung fibrosis.
- **Asthma model findings:** RLN-/- mice subjected to chronic ovalbumin-induced allergic airways disease (AAD) have a more severe airway/lung fibrotic phenotype compared to their wild-type (+/+) counterparts; which can be reversed by systemic [4] or intranasal [6] rH2 RLN treatment. Our collaborator Prof. Schulz (co-CI NHMRC GNT0546428; 2009-11); Institute for Inhalation Biology, Germany) conducted a GWAS in mice and demonstrated an association between four top genes (including *RLN*) and airway hyperresponsiveness (AHR) [9]. Based on this, CIA/CIB have demonstrated a therapeutic role for rhRLX (also known as serelaxin/RLN) in treating the airway remodelling and fibrosis associated with experimental chronic AAD/asthma [4-6].

IPF *in vitro* and mouse model of PF preliminary findings: RLN inhibits TGFβ1-induced upregulation of collagen and fibronectin, and induce a matrix-degrading phenotype in primary human lung fibroblasts [10]. Furthermore, the anti-fibrotic effects of RLN prevent bleomycin (BLM)-induced PF in mice [10], with similar results achieved using synthetic homologs of the RLN peptide [11].

- RLN is an endogenous inhibitor of MLC_{20} phosphorylation and lung fibrosis post-BLM-induced PF – shown by AI Zhou/CIB Samuel. Phosphorylation of MLC_{20} is prominent in both the fibroblastic foci of human IPF and fibrotic regions of BLM-injured mice [12].
- RLN attenuates contraction of myofibroblasts isolated from IPF patient-derived lungs was shown by AI Zhou [12]; who will assist us with measuring myofibroblast contractility.
- **The mechanisms of RLN action are well-established and broad-based:** The anti-fibrotic actions of RLN are related to its ability to interfere with TGF-b1 signal transduction (at the level of Smad2 phosphorylation) [6,13]; and consequently TGFβ1-induced myofibroblast differentiation and collagen production. Furthermore, RLN stimulates matrix metalloproteinase (MMP)-induced collagen degradation [4,10,14], adding to its therapeutic significance as an antifibrotic. Importantly though, RLN does not affect basal collagen [10]; highlighting its safety as a therapeutic.

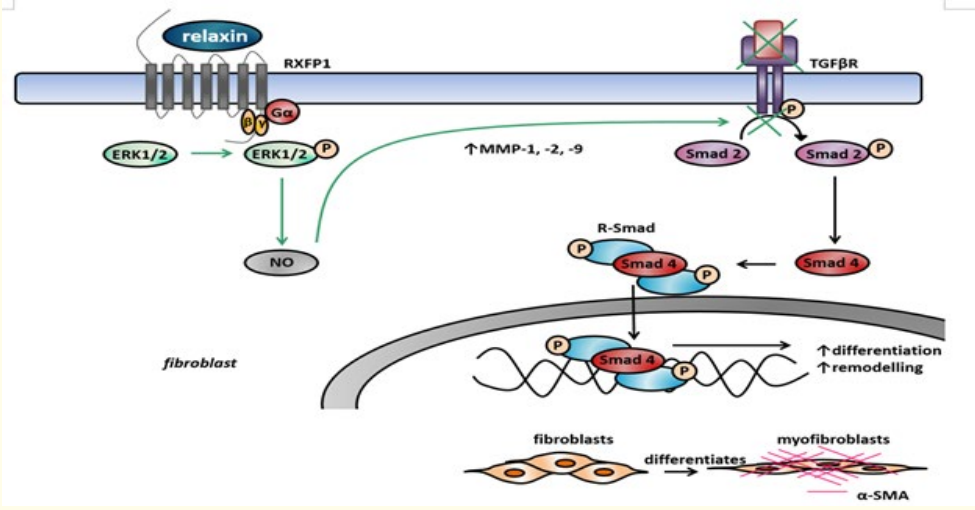

Figure 1: *Mechanisms of RLN action well-established and broad-based.*

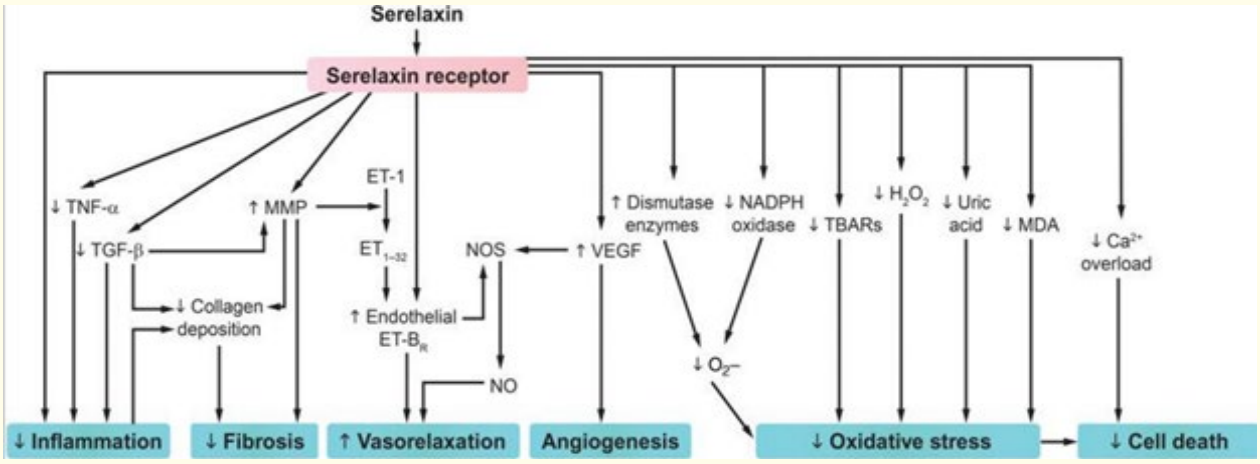

Figure 2: *Other studies/ Useful effects-Bronchodilation effects on lung epithelial cells used with corticosteroids and epithelial repair agent and in epithelial repair model.*

Given these promising findings, we will develop the therapeutic potential of RLN – by evaluating its 1) direct effects in a murine model of BLM-induced PF, with LPS- or TGF- β 1-induced-exacerbations; 2) expression and that of RXFP1 in IPF and lung transplant patients; and 3) effects in a Phase I clinical trial in lung transplant recipients.

Research Plan

Aim-1: A) Evaluate the effects of RLN administration to chronic bleomycin-induced PF, with LPS-induced epithelial damage or TGF- β 1-stimulated exacerbations

Rationale: In both IPF and CLAD there is a significant challenge to treat the lung where the pathogenesis and symptoms originate. Given that more efficacious treatments are required to be delivered over a long period to reverse these chronic diseases, it is vital to use local delivery to limit off-target and systemic side-effects. Successful therapies for lung diseases must reach target tissue throughout the entire lung, passing through the large airways to the periphery and small airways. These have a larger cross-sectional surface area and volume than airways > 2 mm, but only account for 10% of airway resistance. Hence, they constitute a silent zone of ventilation heterogeneity [15]. Therefore it is vital that techniques are used that allow us to study both small and large airways. In this application we will use innovative techniques: surface acoustic waves (SAWs) nebulization to generate aerosols to reach deep into lung.

In order to eventually conduct a clinical trial in IPF patients from diagnosis, a rigorous justification is needed for using RLN in a trial. Therefore preclinical work in animal (Aim 1) and human (Aim 2) models is an essential prerequisite. Several animal models of IPF are available but the gold standard is the murine bleomycin model. This model has both strengths and weaknesses in simulating the human disease and only the most severe bleomycin models are moderate predictors of clinical treatment efficacy. Most important is that the model lacks the following key aspects of human pathology; (i) the correct phenotype including glass opacities by CT; (ii) aging – most animal models are conducted in young and pubescent animals without the oxidative stress-related effects of age (iii) chronicity – the standard model does not involve long-term bleomycin delivery; (iv) exacerbation – the bleomycin models do not include the acute epithelial driven injury that sets off the inflammatory / fibrotic cascade.

CIA/CIB have > 15 years of collaboration in rodent preclinical trials of RLN and other treatments in fibrotic models. We will conduct animal model trials of RLN in two different in vivo models of pulmonary fibrosis which address the limitations of the original BLM models mentioned above. Since aged mice respond differently to fibrotic stimuli such as BLM (which more accurately recapitulates the human

condition [16]); mice will be aged to 12 months before treatment with RLN. Additionally the effects of RLN will be compared to that of Pirfenidone – which is currently on the market as a treatment for IPF.

Approach: Male C57B6J mice (n = 10/group) will be aged to 52 weeks of age and then subjected to BLM (0.15mg) + 1) LPS-induced epithelial damage (BLM-LPS); or 2) +TGF- β 1-induced exacerbation (BLM-TGF) over a 4-week period (from weeks 52 - 56). Following this, mice will be intranasally (i.n)-treated daily with RLN or Pirfenidone, over a 2-week treatment period (a time-point in which RLN has consistently demonstrated its anti-fibrotic effects [4-6]; from weeks 56 - 58).

i) Saline-injected/no-treatment – no-injury control
ii) BLM-LPS/saline-treated – no-injury control
iii) BLM-LPS/RLN-treated
iv) BLM-LPS/Pirfenidone-treated
v) BLM-TGF/saline-treated – no-injury control
vi) BLM-TGF/RLN-treated
vii) BLM-TGF/Pirfenidone-treated

At 58 weeks of age (and after 2 weeks of RLN or Pirfenidone treatment), the following end-points will be measured:

- Death as an endpoint: by Kaplan Meyer plot;
- Lung function: by FlexiVent and invasive plethysmography [4-6];
- Lung inflammation: by differential cell count and inflammation score in H&E [4-6]; Ashcroft score [17];
- Lung fibrosis: by morphometry of Masson trichrome staining [3-6]; hydroxyproline analysis [3-6];
- TGF-beta signal transduction: by ELISA, Smad IHC [6,13];
- Cell apoptosis: by cleaved caspase IHC;
- microCT scan: using Monash Biomedical Imaging facilities.

Aim 1: B) Demonstrate that RLN can be successfully nebulized, retain bioactivity and reach target tissues including the small airways

Rationale: Inhaled delivery has several advantages over oral or systemic delivery including; i) direct to organ targeting, ii) ↓side-effects, iii) ↑ventilation perfusion matching and gas exchange, iv) ↑local drug concentration and ↓drug cost. While i.n. delivery in mice involves pipetting a solution onto the nares where it is inhaled, this is clearly not an effective mode of delivery into patients. In humans, drugs must be delivered via an inhaler or nebulizer to allow fine particles to reach the small airways. Hence, we will develop an innovative method, which is likely to be clinically acceptable means of delivering RLN, by nebulizing RLN such that it (i) retains bioactivity and (ii) reaches receptors (i.e.RXFP1) in the bronchial tree (including small airways).

Conventional ultrasonic nebulizers generate readily respirable fine mists but the energy they impart to solutions leads to protein damage. Acoustic wave microfluidic technology, developed by CIC Yeo (Micro/Nanophysics Research Lab, RMIT University), does not overheat or shear proteins [18]. These devices use surface acoustic waves (SAWs) to generate aerosols with excellent particle distributions (Figure 3). SAW requires two orders of magnitude less energy to generate sprays meaning that technology is ideal for a rechargeable battery powered portable hand held device. Another advantage is the low energy imported to the fluid phase, which does not damage delicate protein structure. This technology is under clinical development for human use by CIC Yeo [18]. Control TGF- β 1 (T) T + rhRLN T + Neb rhRLN A B. (A) Nebulized RLN maintains bioactivity – RLN was nebulized via microfluidic nebulizer at CIC Yeo's Lab at RMIT University, collected and added to human airway fibroblasts seeded at a density of 100,000 cells/12-well plate well. After 72h supernates were collected and (collagen-degrading) MMP-2 was evaluated by gelatin zymography and densitometric analysis. Fibroblasts treated with nebulized RLN (60 ng/ml ~ 10 nM) had equivalent MMP-2 expression as those treated with RLN that was added from stock – not nebulized. (B) Particle size distribution of a similar peptide to RLN is excellent.

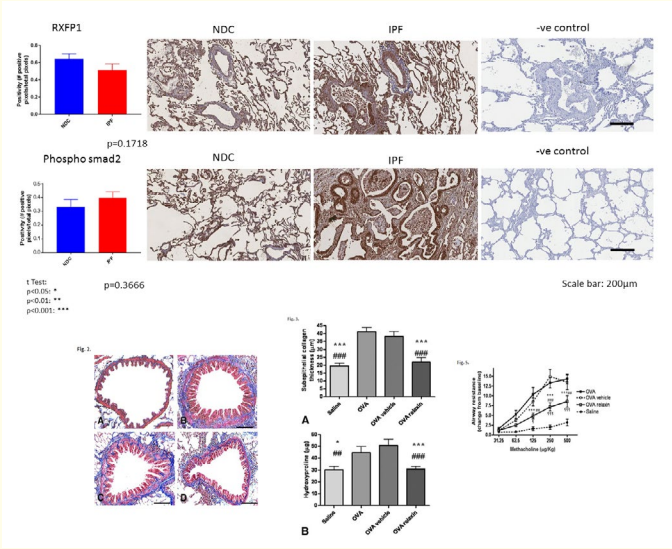

Figure 3: *Established peribronchial fibrosis can be reversed by systemic rH2 RLN treatment. This can also be achieved using daily I.N administration of RLN.*

Preliminary data

Approach

***In vitro* studies:** To first confirm that nebulized RLN is bioactive, we will add nebulised RLN to diseased and donor human lung fibroblasts (obtained from the ALF Biobank; AI Westall) and evaluate end-points that are known to be stimulated by RLN as part of its anti-fibrotic actions, including (n = 5 separate times per end-point): i) pERK1/2; ii) nNOS; iii) pSmad2; iv) α -SMA; v) MMPs; and vi) type I collagen – from the supernatants and/or cell lysates; by Western blotting and zymography [19].

We will also examine the bronchodilatory effect of nebulized RLN on human and rodent airways, arteries and lung slices [20] under conditions established in Aim 1A to induce AHR and altered vascular responsiveness (n=5 separate times per end-point).

***In vivo* studies:** Using aged mice as described in Aim-1A; we will establish the same treatment groups as outlined on page 4 – but will replace i.n RLN or Pirfenidone with nebulized RLN or Pirfenidone. For comparison we will use normal stock RLN (without nebulization) and RLN put through conventional ultrasonic nebulizers (Aeroneb, Omron). The same end-points outlined on page 4 will then be measured

Expected outcomes: These studies will establish that RLN is suitable to progress to trials in humans for treatment for IPF and CLAD; and demonstrate the potential of delivering RLN to reach the small airways and have therapeutic effects.

Aim-2: A) Characterize relaxin and RXFP1 expression in patients with IPF and transplant recipients Rationale: The development of techniques to accurately and precisely measure levels of RLN and RXFP1 in clinically relevant patient samples (BALF, blood) is important for a number of reasons:

1. Given the role of endogenous RLN in regulating fibrosis, and our findings of a reduction of RLN protein expression in endobronchial biopsies from patients with severe asthma and airway remodelling, endogenous RLN levels may be of relevance to RLN therapies in post-lung transplant and in IPF. Furthermore, RLN has potential as a biomarker for a range of fibrotic diseases. GRANT PROPOSAL – 2017 Project Grants Application ID: APP1139749 CIA Surname: Royce.

2. There is interest in RXFP1 levels and whether these might affect RLN-based therapies in IPF [21]. However, these have only been measured by mRNA which has only limited functional relevance. Protein measurements of RXFP1 are needed; as discussed by us [22]. Again measurement of RXFP1 might be useful in other disease contexts – for instance RELAX-AHF [1,2].

3. Commercially available assay kits for RLN and RXFP1 are available, they have not proven to be reliable measures of the peptide and the need for validation of these kits was emphasized at the last International Relaxin Conference (2015). The weakness of these kits is the specificity of the detection antibodies and capture antibodies (in the case of sandwich ELISAs). Peptides similar to RLN are present in biological systems (i.e. RLN homologs -H1 and H3, relaxin-like peptides, as are other structurally-related members of the insulin superfamily; (i.e. RXFP2 and related g-coupled protein receptors), and therefore antibody cross-reactivity is a major issue.

Approach: BAL fluid collected from IPF patients (n = 10), transplant recipients (n = 10) and normal donors age and sex matched (n = 10) as well as patients from Aim 3 will be acquired from The Alfred Hospital Lung Biobank (Director, AI Westall). Each patient/donor sample will be separated into BALF supernatant (15 - 30 ml) and BALF cells (1 million cells per mL) by centrifugation.

5 ml of supernatant and 1 million cells will be transported to the Monash Biomedical Proteomics Facility (Director, AI Schittenhelm). The supernatants will be used for RLN measurement (as RLN is a secreted protein), whilst the cells will be used for RXFP1 analysis. Briefly, proteins present in the BALF (containing RLN) or isolated from the cells (containing RXFP1) will be tryptically digested to peptides and analysed by LC-MS/MS on a QExactive Plus mass spectrometer (Thermo Scientific) to obtain spectral information (for example exact mass, fragmentation pattern, retention time) of RLN- and RXFP1-derived peptides. Subsequent analysis of these peptides on a SCIEX 6500 QTrap mass spectrometer operated in multiple reaction monitoring (MRM) mode will allow us to absolutely quantify RLN concentrations in BALF supernatants and RXFP1 in these cells. Very similar methods have been used by AI Schittenhelm to successfully identify renin angiotensin (RAS) peptides in inflammatory bowel disease plasma samples in 2016. This was part of a similar collaboration between CIA and Drs Gibson/Lubel/Garg Monash Gastroenterology funded by Broad Foundation USA. This was a significant achievement given that the RAS peptides are small and differ only by 1 - 4 amino acid, highlighting the skill of AI Schittenhelm's team and the strength of this technology available to the Monash Proteomics Platform regarded as the best in the country.

Commercially available ELISA kits for RLN and RXFP1 (Immunodiagnostiks) will be obtained and validation of these using the same samples (detailed above); in comparison to synthetic RLN peptides and homologues as positive controls, along with the kit standards. CIA has already designed ELISA plate formats for kit validation and performed validation of renin angiotensin peptide ELISA kits for the Broad Foundation study.

Expected outcomes: A method for accurately measuring RLN and RXFP1 will be developed. The validity of commercially available kits will be determined definitively. This will have massive interest for the RLN research community; for clinical trials being undertaken for Serelaxin and synthetic forms of RLN as a therapeutic (heart failure, scleroderma); and has possible biomarker implications in fibrotic diseases, which is pertinent to the current proposal.

(Aim 2Aii) Histology FFPE study

Rationale: Characterization of localization of protein expression of RXFP1 and RLN and other relevant proteins in the RLN ECM regulatory cascade, and their relation with key markers of IPF and post-transplant airway remodelling is vital for this proposal and complements the other investigations in Aim 3. We are fortunate to have access to FFPE from > 10 IPF patients, > 10 donors and > 5 post-transplant care of AI Westall/ALF Biobank. The number of samples available will continue to grow during the course of the project.

Approach: IHC and histology: Serial sections will be stained using monoclonal antibodies to the markers below. Stained sections will be scanned into the Leica Aperio (Monash Histology Platform) and available for telepathology and intensity and extent of staining will be assessed using the inbuilt Aperio algorithms. Histochemically stained sections will be scored for standard IPF endpoints. A histopathologist will be available for validation. Correlations will be performed between results. Colocalization studies will be performed using IF confocal.

Histoindex visualization of collagen: (A) normal collagen around airways in donor, (B) fibrotic consolidation of lung tissue - IPF, (C) airway remodelling typical of CLAD.

Histoindex: A Histoindex machine has been installed in the Fibrosis Laboratory (Monash Pharmacology; Head, CIB), obtained through a Monash University Equipment Grant (Samuel., *et al*. 2017). The Histoindex is able detect collagens and other fibrillary proteins using 2nd harmonic technology and lasers at various wavelengths. CIB and CIA are in close contact with the Singapore-based developers of the Histoindex platform, which has already prepared algorithms for the detections and quantification of fibrotic lesions in PF models used by a number of Pharma clients. Parameters including interstitial and total collagen area, vascular collagen area, collagen fibre thickness etc. can be measured. We will scan unstained sections from all available cases. This will be the first time the technology will be used in this disease setting (see Histoindex-produced images above; A-C).

- Changes in Masson-trichrome and Histoindex-detected collagen will be co-localised with expression of RLN; RXFP1; α-SMA; TGF-β1; MUC5b; TERC/TERT; MLC20;
- Ashcroft scores of inflammation and fibrosis [17]; PCNA [23] and Ki67 [24] staining-associated cell proliferation; markers of type II epithelial cells (SPC, SPA); ABPAS-staining of goblet cells [4-6]; detection of Clara cells [24], EMT markers (loss of e-cadherin/ increase in α-SMA-associated myofibroblasts); fibrocyte markers, endothelin system (ET1, ETRB, ETRA); trefoil family (TFFs), CAV1 [25]; mucins (MUC5B etc); macrophage markers (M1, M2) will also be measured.

Outcomes: This study will provide a thorough and complete histological and immunohistochemical characterization of IPF in comparison to donor controls and post-transplant lungs, with a particular emphasis on the RLN/RXFP1 system and important markers of disease progression as well as epithelial and fibroblast cell phenotype. We will also use the power of the Histoindex platform for quantification and characterization of the lungs.

Aim-2: B) Determine the effects of RLN treatment on markers of IPF-induced fibrosis using IPF patient and donor control lung fibroblasts *in vitro*

Rationale: Animal models have certain limitations particularly with regard to emulating the idiopathic nature of IPF. Whilst very similar, mouse cells are not genetically/proteomically identical to human cells. Additionally, human cells from IPF patients may have genetic and epigenetic differences to cells from normal humans or donors, therefore it is essential these are used and compared with donor controls in cell culture experiments. In as much as it forms the bulk of the fibrotic UIP lesions, the effector cell in IPF is the fibroblast. We will concentrate on this cell type in our studies as it is relatively easy to grow. However, we will use cell culture models that attempt to simulate various aspects of the fibrotic milieu *in vivo* such as stimuli related to the key aspects of (i) fibrotic growth signals, (ii) epithelial injury signals, (iii) aging signals as a test bed for serelaxin. Our endpoints will be assess (i) fibrosis – ECM deposition, (ii) myofibroblast differentiation, (iii) fibrotic signalling, (iv) migration and invasion. These endpoints are highly descriptive of severity and importantly progression.

Approach: Lung fibroblasts from IPF patients (n = 10 patients) and donors (n = 10) age/sex matched donors will be obtained from those archived at the Alfred Lung Biobank (Director, AI Westall) and used for all experiments described below.

Stimuli to simulate the microenvironment in the IPF lung:

1) Fibrotic Growth Signals: Cells will be stimulated with human recombinant TGFβ1 (5 - 10 ng/ml) [26]; PDGF-BB (50 ng/ml); FGF-basic (20 ng/ml); or EGF (3 ng/ml) for stimulation of proliferation;
2) Epithelial Injury Signals: Cells will be stimulated with BALF from IPF (sterilized by filtration with a 22 μm filter);
3) Aging Signals: Hydrogen peroxide (H_2O_2) treatment will simulate the high oxidative stress microenvironment associated with senescence-like growth arrest [27]. AI Kalionis used H_2O_2 treatment to induce oxidative stress in mesenchymal stem/stromal cells (MSCs) to simulate the microenvironment of MSCs affected by preeclampsia [28]; characterised by high levels of oxidative stress, cell senescence and ageing.

Treatment: All experiments will be treated with 1 - 100 ng/ml Serelaxin over 48-72h.

Endpoints: Supernatant and cell lysate samples will be collected and used for the following assays. The remainder will be archived for use on other IPF and remodelling markers that may be of interest.

- **Proliferation:** By the MTA assay;
- **Collagen deposition (fibrosis):** by the Sircol colorimetric assay which can be performed rapidly on small samples. Findings will be confirmed using the hydroxyproline (HP) assay [29];
- **Myofibroblast differentiation:** by Western blotting [29] and immunofluorescence staining for α-SMA; **myofibroblast contractility:** by the wrinkle assay and phosphorylated MLC20 expression [12];
- **TGFb1 expression:** by ELISA and Western blotting [19];
- **Invasion assay:** Invasion of fibroblasts into surrounding lung tissue is a key feature of IPF. AI Kalionis has developed an invasion assay (xCELLigence real-time cell functional assay);
- **Migration assay:** The xCELLigence system will also allow time related tracking of migration across the different experimental groups.

Aim-3: Evaluate the effects of RLN in a Phase I clinical trial in lung transplant recipients

Rationale: Peribronchial fibrosis (airway remodelling) is a common feature in CLAD and is associated with rejection. It is also a feature of asthma, where it is associated with AHR. In chronic mouse models of airway remodelling, we have repeatedly shown that RLN can normalize remodelling, reverse fibrosis and reduce AHR (in response to methacholine-induced bronchoconstriction) [4-6]. We therefore hypothesize that RLN may have similar effects in patients. Another feature of CLAD is OB in the small airways; a fibrotic lesion that may also be a good target for RLN.

Approach – Phase I Clinical Trial: Eight patients will be recruited from the lung transplant unit of The Alfred Hospital, Melbourne (coordinated by the Director, CID). Recruitment will begin in Year 1 to ensure sufficient time for recruitment and completion of all experiments. All participants will provide written informed consent before commencement of the study. The study protocol will be approved by the Alfred Health and Monash University Human Research and Ethics Committees. The Clinical Trial will be conducted as per the model established by CID and used in previous trials he has conducted [30]. A detailed eight page Study Protocol has been prepared by CID. In brief it outlines: Eligibility criteria (inclusion, exclusion criteria, concomitant and intercurrent medication); Clinical material to be collected (including BAL)and lung function testing:

- **Full Study Schedule** (including screening, enrolment, baseline investigations, bronchoscopy, schedule of administration, evaluation during study, study procedures; and
- **Detailed schedule of visits:** Relaxin administration (via intravenous infusion as per the RELAX AHF protocol1 – which has demonstrated safety) will be performed at baseline, with followup visits at day 18 +/-3d, day 42+/-5d, day 90+/- 5d, day 180+/-5d, and day368+/-14d.

Endpoints

Primary endpoint: Progression-free survival at 12 months. This will be defined as a composite of all-cause mortality and freedom from CLAD progression (defined as fall in FEV_1 > 10% from the baseline (Screening Visit) FEV_1) at 12 months.

Secondary endpoints: All-cause mortality at 12 months; CLAD-related mortality at 12 months; Freedom from CLAD progression (defined as fall in FEV_1 > 10% from the baseline (Screening Visit) FEV_1) at 12 months; Time to fall in FEV_1 > 10% from the baseline (Screening Visit) FEV_1; Freedom from OB grade 3 at 12 months; Slope of FEV_1 decline at 12 months; Change in St George's Respiratory Questionnaire at 6 and 12 months; Inpatient bed days; Health care costs.

Safety endpoints: Incidence of infections requiring treatment at 12 months; Incidence of malignancy at 12 months.

Exploratory endpoints: Change in bronchoalveolar lavage (BAL) neutrophil count, IL8, TGF-β1, IL6, IL10 and TNFα.

Expected outcomes: This study will for the first time establish the safety of RLN delivered to the lung of patients. The range of endpoints examined and analysis methods are likely to be able to detect any effects of RLN on remodelling and bronchial hyperresponsiveness to MCh.

Power calculations and statistical tests: Based upon previous studies, minimum experimental sample sizes of n = 10 rodents are required to provide 80% power at α value of 0.05 to detect differences between groups for mechanics and reactivity [4,6,31]. This takes into account estimated 20% loss. Statistical tests: 2 way ANOVA with Bonferonni post-test for lung function analysis and Mann Whitney and t-tests for the analyses of tissue end-points.

Significance and Innovation

The current proposal has the potential to improve the survival of Australians afflicted by two of the most debilitating conditions – IPF and CLAD. Lung transplantation, is per patient one of the most expensive procedures in our healthcare system and despite the high quality of care in Australia's leading transplant units, acute and chronic rejection is still very common. The current proposal has high significance in that it has the potential to reduce the need for lung transplants by improving the prognosis for IPF patients as well as improving the prognosis post-transplant. In addition to prolonging life, there is potential for improving the quality of life. Moreover, there is potential for reducing the need for other treatments by saving normal lung tissue from scarring. There is also the prospect of restoring, partially or completely, normal organ function as well as ameliorating lung function via bronchodilation. These would represent significant advances in the context of these diseases, drug therapies currently under development are not effective, and at best slow inevitable disease progression.

The innovation of this proposal is the repurposing of serelaxin, a safe and well-established antifibrotic to IPF/CLAD, as well as the quality of the design and choice of models and techniques. This will complement and strengthen the impact of the anticipated results to ensure publication in top-tier journals. The models (including consideration of an aging profibrotic milieu) in humans *in vivo*, murine models *in vivo* and human *in vitro* systems with a combination of standard clinical endpoints (pathology, CT, LF) and novel (invasive assay, 2nd harmonic, microfluidic delivery, proteomic investigations) using the best CI and AI collaborations in the areas studied will result in a strong an highly feasible research program with high likelihood of future clinical application. GRANT PROPOSAL – 2017 Project Grants Application ID: APP1139749 CIA Surname: Royce

Discussion

Relaxin has been shown to exhibit strong anti-fibrotic effects in a myriad of human fibroblast culture models where TGF-β1 is used as the profibrotic stimulus to initiate myofibroblast differentiation and collagen synthesis [2] and animal models where TGF-β1 is upregulated by chronic inflammation [3].

Furthermore, in a previous study using human IPF-derived lung myofibroblasts, there was decreased MLC_{20} phosphorylation and reduced contractility in response to human recombinant relaxin [4].

Additionally, in this study and others, relaxin (or related analogues) has been shown to prevent and more importantly reverse established lung fibrosis in bleomycin-induced models of pulmonary fibrosis *in vivo* (ameliorating Ashcroft scores and aberrant lung collagen deposition) [2].

Relaxin has also been shown to have similar therapeutic effects in numerous chronic models of airway remodeling, COPD and others characterized by established pulmonary fibrosis [3,5]. So given this more than circumstantial evidence, how are the human IPF findings explained?

One of the main sites of expression of RXFP1 is the bronchial epithelium [6] and that epithelial cells are highly secretory and may be highly influential on the underlying mesenchymal cells including fibroblasts and myofibroblasts via cytokines and growth factors.

Interestingly, when systemically administered to a mouse model of chronic allergic airways disease (in which RXFP1 expression was decreased compared to that in saline-treated controls), human relaxin increased RXFP1 expression in the airway epithelium [6].

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