Role of Human Antigen R (HuR) in Lung Fibroblast Differentiation: Implications for Idiopathic Pulmonary Fibrosis

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April 2020

A Thesis Submitted to McGill University in Partial Fulfillment of the Requirements of the Degree of Master of Science

Acknowledgments

I would like to take this time to express my deepest gratitude to both of my supervisors, Dr. Carolyn Baglole and Dr. Ilan Azuelos, for their unwavering guidance and encouragement. Their continuous belief and support helped me to be more confident and pursue my research project with focus, discipline and excitement. I would also like to thank previous and current laboratory colleagues for contributing to a positive work environment and a willingness to always provide assistance: Hussein Traboulsi, Nouf Aloufi, Fatimah Alhabeeb, Necola Guerrina, Kashmira Prasade, Max Liu, Fangyi Shi, and Matthew Preteroti. Each in their own way has inspired me with their dedication and hard word, to say nothing of their collegiality and kindness. I would also like to acknowledge the faculty members of the Meakins-Christie Laboratories, in particular Dr. David Eidelman, for his valuable scientific advice. Thank you to Andree Gravel in the Drug Discover Platform at the RI-MUHC for her technical expertise with ^{1}H NMR. Finally, I would like to thank my family and friends for their support and encouragement during my journey. It would have not been possible without them.

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LIST OF ABBREVIATIONS

Abbreviation	*Meaning*
ACTA2	Actin alpha 2, smooth muscle gene
α-SMA	Alpha smooth muscle actin
AEC	Alveolar epithelial cell
ANOVA	Analysis of variance
ARE	AU rich element
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid
BLM	Bleomycin
Chk2	Checkpoint kinase 2
COL1A1	Collagen type I alpha 1 chain gene
COX-2	Cyclooxygenase 2
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epithelial growth factor
ELAV	Embryonic lethal abnormal vision
ELAVL-1	Embryonic lethal abnormal vision-like protein 1
EMT	Epithelial-mesenchymal transition
EPC	Endothelial progenitor cells
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FDG	^{18}F-fluorodeoxyglucose
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FMD	Fibroblast to myofibroblast differentiation
FN1	Fibronectin 1 Gene
FVC	Forced vital capacity
GLUT-1	Glucose transporter 1
GWAS	Genome wide association studies
HIF-1α	Hypoxia inducible factor 1 alpha
HKII	Hexokinase II
HK2	Hexokinase 2 Gene
HLF	Human lung fibroblast
HNS	HuR nucleocytoplasmic shuttling sequence
HRCT	High-resolution computed tomography
HSC	Hepatic stellate cell
HuR	Human antigen R
HRP	Horseradish peroxidase
IF	Immunofluorescence
IGFBP-5	Insulin-like growth factor binding protein 5
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
KSRP	KH-type splicing regulatory protein
LAP	Latency associated peptide
LDH	Lactate dehydrogenase
LDHA	Lactate dehydrogenase A
LLC	Large latent complex

LTBP	Latent TGFβ1 binding protein
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential media
miRNA	MicroRNA
MRTF	Myocardin related transcription factor
^{1}H NMR	Proton nuclear magnetic resonance
ncRNA	Noncoding RNA
NPM	Nucleophosmin
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
PFK	Phosphofructokinase
PK	Pyruvate kinase
PKC	Protein kinase C
qPCR	Quantitative polymerase chain reaction
RBP	RNA-binding protein
RRM	RNA recognition motifs
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SG	Stress granule
siCtrl	Small-interfering Control
siHuR	Small-interfering HuR
siRNA	Small-interfering RNA
SLB	Surgical lung biopsy
SRF	Serum response factor
TCA	Tricarboxylic acid
TGFβ1	Transforming growth factor beta 1
TIN2	Telomere associated protein 2
TNF-α	Tumor necrosis factor alpha
TSP	Total soluble protein
Type I AEC	Type I alveolar epithelial cell
Type II AEC	Type II alveolar epithelial cell
UIP	Usual interstitial pneumonia
UPR	Unfolded protein response
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive fibrotic lung disease affecting an increasing number of people worldwide. Although the precise factors that cause IPF are still unknown, the disease pathogenesis is proposed to be driven by recurrent alveolar epithelial injury which leads to the differentiation of fibroblasts into α-smooth muscle actin (α-SMA)-expressing myofibroblasts (FMD, fibroblast-to-myofibroblast differentiation) and a subsequent excessive and relentless deposition of a collagen-rich extracellular matrix (ECM). Transforming growth factor beta-1 (TGFβ1) plays a key role in the process of FMD and is thought to be the most important pro-fibrotic mediator involved in the pathogenesis of IPF. Although the exact mechanisms that regulate FMD have not yet been fully elucidated, the RNA-binding protein Human antigen R (HuR) translocates to the cytoplasm in response to TGF-β1, where it stabilizes various profibrotic mRNAs. Interestingly, cytoplasmic HuR also stabilizes and increases the translation of the hypoxia inducible factor (HIF-1α) mRNA which encodes a transcription factor critical for inducing a metabolic shift from oxidative phosphorylation towards glycolysis, thereby increasing lactate levels under hypoxic conditions. This metabolic shift may be an important factor in activating TGFβ1, thereby inducing FMD. As one of the hallmark features of tissue fibrosis is the presence of a hypoxic microenvironment, we hypothesize that under hypoxic conditions, HuR regulates mRNAs and proteins crucial to the process of FMD and glycolytic reprogramming in human lung fibroblasts. The aims of this study are: (1) investigate the effect of hypoxia alone and in combination with TGFβ1 on metabolic reprogramming and myofibroblast differentiation**;** (2) assess the effect of hypoxia alone and in combination with TGFβ1 on HuR expression and subcellular localization; and (3) investigate the role of HuR on metabolic reprogramming and myofibroblast differentiation. First, by using qPCR, western blot and proton nuclear magnetic resonance spectroscopy (^{1}H NMR), we found that hypoxia alone did not have significant effects

on FMD or metabolic reprogramming. However, hypoxia reduced markers of TGFβ1-induced FMD and glycolytic reprogramming in human lung fibroblasts (HLFs). While hypoxia- together with TGFβ1- increased mRNA levels of differentiation and glycolysis, such as *ACTA2, LDHA,* and *HK2*, we found that protein levels of α-SMA, collagen 1 and lactate were significantly reduced, indicating a decrease in FMD and glycolytic shift. Levels of HuR mRNA and protein did not change in response to hypoxia with or without TGFβ1. However, using immunofluorescence (IF), we found that hypoxia, TGFβ1 and TGFβ1 combined with hypoxia induced cytoplasmic translocation of HuR- a feature consistent with HuR activation. In order to confirm whether HuR is necessary to the process of FMD and metabolic reprogramming, HLFs were transfected with HuR siRNA (siHuR) or control (scramble) siRNA (siCtrl) treated with hypoxia, TGFβ1 and TGFβ1 combined with hypoxia. We found that siHuR-transfected cells had significantly reduced protein levels of α-SMA and collagen 1. However, siHuR did not change levels of lactate. In conclusion, we found that HuR knock-down leads to decreased levels of α-SMA and collagen 1. In addition, our research revealed that hypoxia reduces TGFβ-induced FMD and glycolytic reprogramming. This study is the first to investigate the role of HuR in FMD and metabolic reprogramming in response to hypoxia and TGFβ1, which could help provide the basis for new targeted therapy in fibrotic disease such as IPF.

RESUMÉ

La fibrose pulmonaire idiopathique (FPI) est une maladie pulmonaire fibreuse chronique et progressive affectant un nombre croissant de personnes dans le monde. Bien que les facteurs précis qui causent l'IPF soient encore inconnus, Il est vraisemblable que la maladie est provoquée par des lésions épithéliales alvéolaires récurrentes conduisant la différenciation des fibroblastes en myofibroblastes. Ce processus nommé DFM, (différenciation fibroblastes en myofibroblastes) et un dépôt ultérieur excessif d'une matrice extracellulaire riche en collagène (ECM) sont parmi les caractéristiques de cette maladie. Le facteur de croissance TGFβ1 (pour *Transforming growth factor beta-1*) joue un rôle clé dans le processus dans la fibrose pulmonaire et il est considéré comme le médiateur profibrotique le plus important impliqué dans la pathogenèse de l'IPF. Les mécanismes exacts qui régulent la DFM ne sont pas encore entièrement élucidés. Cependant, il a été démontré que la protéine de liaison à HuR (pour *Human Antigen R*) se transloque vers le cytoplasme où il est connu de stabiliser divers ARNm, incluant les ARNm codants pour des protéines profibrotiques. D'une manière intéressante, il a également été prouvé que HuR, une fois dans le cytoplasme, il stabilise et augmente la traduction de l'ARNm codant pour HIF-1α (pour *Hypoxia inducible factor alpha-1*). Ce dernierest un facteur de transcription important qui coordonne l'induction de plusieurs gènes et processus physiologiques et métaboliques (par exemple: stimulation la glycolyse anaérobie et l'éry-thropoïèse) qui concourent à compenser la rareté d'oxygène connu dans les tissus. HIF-1α donc, et en conditions d'hypoxie, il induit une transition métabolique de la phosphorylation oxydative vers la glycolyse. Ce qui augmente ainsi les niveaux de lactate. Cette transition métabolique pourrait jouer un rôle important dans l'activation du TGFβ1, et par suite stimulant la DMF. Comme l'une des caractéristiques de la fibrose tissulaire est la présence d'un microenvironnement hypoxique, nous émettons l'hypothèse que durant le développement de la fibrose pulmonaire, HuR régule les ARNm et les protéines

cruciaux pour le processus jouant un rôle dans la différentiation des fibroblastes ainsi que dans la reprogrammation glycolytique. Les objectifs de cette étude sont: (1) étudier l'effet de l'hypoxie seule et en combinaison avec TGFβ1 sur la reprogrammation métabolique et la différenciation des myofibroblastes; (2) évaluer l'effet de l'hypoxie seule et en combinaison avec TGFβ1 sur l'expression de HuR et sa localisation subcellulaire; et (3) Investiguer le rôle de HuR sur la reprogrammation métabolique et la différenciation des myofibroblastes. Premièrement, en utilisant la PCR quantitative (qPCR), la technique de western blot et la spectroscopie de résonance magnétique nucléaire (RMN), nous avons constaté que l'hypoxie seule n'avait pas d'effets significatifs sur la DMF ou la reprogrammation métabolique. Cependant, l'hypoxie a réduit les marqueurs de la différentiation induite par TGFβ1 et la reprogrammation glycolytique dans les fibroblastes pulmonaires humains (FPH). L'addition du TGFβ1 en présence de l'hypoxie,a augmenté l'expression des ARNm de différenciation et de glycolyse, tels que ACTA2 *(*Pour *Actin Alpha 2, Smooth Muscle)*, LDHA *(*Pour *Lactate dehydrogenase A)* et hexokinase 2 (HK2*)*. À notre surprise, et contrairement à l'ARNm correspondant, nous avons observé que les niveaux de protéines:α-SMA (Pour *alpha smooth muscle actin*), de collagène 1 et de lactate étaient considérablement réduits. Ce qui suggère que l'hypoxie cause une diminution de la différentiation des fibroblastes et des changement métaboliques induites par TGFβ1. Toutefois, les niveaux d'ARNm et protéiques d'HuR n'ont pas changé en dans toutes les conditions. Cependant, et en utilisant l'immunofluorescence (IF), nous avons démontré que l'hypoxie, TGFβ1 et TGFβ1 combinée à l'hypoxie induisent une translocation cytoplasmique de HuR. Ceci est une caractéristique compatible signifie l'activation de HuR. Dans le but de confirmer que HuR joue un rôle dans le processus de DMF et la reprogrammation métabolique, nous avons transfecté les FPH avec petits ARN interférents (pARNi) dirigé contre HuR (piHuR). Ensuite, nous avons exposées ces cellules soit à l'hypoxie seule, au TGFβ1 seul ou à la combinaison des deux. D'une manière

intéressante, nous avons constaté que les cellules transfectées par piHuR avaient des niveaux de protéines significativement réduites d'α-SMA et de collagène 1. Cependant, l'absence de HuR n'a pas changé les niveaux de lactate. En conclusion, nous avons constaté que l'inactivation partielle de HuR démontrent les niveaux réduits de α-SMA et de collagène 1. De plus, nos recherches ont révélé que l'hypoxie réduit la DMF induite par le TGFβ1 et la reprogrammation glycolytique. Cette étude est la première à investiguer le rôle de HuR dans la DMF et la reprogrammation métabolique en réponse à l'hypoxie et au TGFβ1. Ce travail pourrait donc constituer la base d'une nouvelle thérapie ciblant HuR dans le but de traiter les maladies fibrotiques telles que la FPI.

Chapter 1 INTRODUCTION

1.1 Interstitial Lung Disease (ILD) and Idiopathic Pulmonary Fibrosis (IPF)

1.1.1 ILD and IPF: definition, epidemiology, healthcare burden

Interstitial lung diseases (ILDs) are an array of lung diseases that involve varying degrees of inflammation and fibrosis of the lung parenchyma.[1,2] Causes for ILD include a variety of environmental, avocational, occupational and medication-related exposures or may result from systemic autoimmune or connective tissue diseases.[1] The most common form of ILD is known as idiopathic pulmonary fibrosis (IPF), a chronic, progressive disease distinguished by abnormal accumulation of fibrotic tissue in the parenchyma of the lungs.[2,3] IPF is a disease of unknown etiology and is associated with an overall poor prognosis and significant morbidity.[2] While several early reports in German-language literature depict autopsy findings to be consistent with the contemporary view of IPF, Hamman and Rich are generally considered to be the first physicians to describe IPF as a pathological entity between 1933 and 1944.[4] As a result of their detailed description on clinical and pathological features of the disease, worldwide recognition of IPF led physicians and researchers to study its cause and pathogenic mechanisms.[4]

Today, it is estimated that the incidence of IPF throughout Europe and North America ranges between 2.8 and 19 cases per 100,000 persons per year.[5] In Canada, the broad prevalence of the disease is estimated at 41.8 per 100,000 while the incidence rate is estimated at 18.7 per 100,000 using national administrative data from 2007-2011 to identify IPF cases in all age categories.[6] The prevalence and incidence were found to be higher in men.[6] In contrast, in Asian countries such as South Korea, Taiwan and Japan, the incidence rates for IPF are amongst the lowest in the world, with estimates ranging from 1.2 to 4.2 per 100 000 people per year.[5] It remains unclear if IPF is more common in Canada or if estimates in other countries around the world vary as a result of

inconsistent case definitions and limited data availability.[6] Nevertheless, the prevalence of IPF appears to increase with age as diagnosis typically occurs in patients over 50 and in a higher proportion of males than females.[5] IPF is a deadly disease that has significant impact on quality of life and overall mortality[7]. Patients typically progress towards respiratory failure as the median survival is 3-5 years following diagnosis. This survival rate is significantly lower than many cancers.[7] From an economic perspective, healthcare costs and resource utilization are increased in patients several years prior to the initial diagnosis.[8] The estimated cost per patient is $2,721 and $7,049 for 5- and 2-years prior to diagnosis, respectively. This economic strain remains elevated as costs per patient total $12,978 and $8,267 at 2- and 3-years following diagnosis.[8] Taken altogether, low survival rates and high healthcare burden galvanize the need for a more comprehensive analysis of IPF incidence and prevalence worldwide to accelerate research into this deadly disease.

1.1.2 IPF characteristics, pathological features and clinical diagnosis

The histological and/or radiological pattern that defines IPF is known as usual interstitial pneumonia (UIP) which leads to worsening of dyspnea and lung function that is associated with a general poor prognosis.[9] This disease primarily occurs in the sixth and seventh decades of life, is limited to the lungs, and should be considered when dealing with adult patients that present with unexplained chronic dyspnea, cough, bibasilar inspiratory crackles and digital clubbing.[9,10] In some rare cases, patients may experience an acute exacerbation as an initial manifestation, such as worsening of dyspnea over a few weeks.[9] The majority of patients suffering from IPF have a history of cigarette smoking although several other risk factors have been linked with the disease such as gastroesophageal reflux, chronic viral infections (Epstein Barr virus, hepatitis C) and a familial history of ILD.[9] Another component of IPF is the existence of several co-morbidities,

which include emphysema, lung cancer, pulmonary hypertension, sleep apnea and coronary heart disease.[10]

One of the primary tools used in the diagnosis of IPF is the high-resolution computed tomography (HRCT), which permits enhanced image resolution following volumetric scanning of the lungs.[9,11] This tool improves the detection of all abnormities even when they are subtle and provides the precise analysis of lesion characteristics.[11] The HRCT features that are associated with the UIP pattern in IPF include honeycombing, traction bronchiectasis and traction bronchiolectasis which may present alongside the occurrence of ground-glass opacification.[9] The presence of honeycombing is defined as clustered airspaces, typically 3-10 mm in diameter, with thick and visibly defined walls. Honeycombing can be viewed as multiple layers of subpleural cysts on top of each other, however it can also present as a single layer.[12] This can make the observation of honeycombing challenging as it mimics the pathology of paraseptal emphysema, traction bronchiolectasis and subpleural cysts.[13] An additional key HRCT feature is traction bronchiectasis/ bronchiolectasis which is described as the subtle irregularity of the bronchial/bronchiolar wall to well-defined airway distortion and varicosity.[14] Finally, the presence of ground-glass opacification is linked with hazy increased opacity of the lung in conjunction with preserved bronchial and vascular borders.[15] The diagnostic criteria for IPF incorporates the key HRCT patterns within 4 categories: 1. UIP pattern, 2. probable UIP pattern, 3. indeterminate UIP pattern, and 4. alternative diagnosis. The UIP pattern contains the presence of distinguishable honeycombing and may or may not be accompanied by traction bronchiectasis/bronchiolectasis. The distribution of UIP is typically subpleural with the majority found at the basal level, although it can be present in the upper lobe. Ground glass opacification may also be present and is usually accompanied by a superimposed fine reticular pattern. The distinction in the case of probable UIP can be

characterized by the absence of honeycombing by HRCT in the presence of peripheral bronchiectasis/bronchiolectasis and potential ground-glass opacification. The category of indeterminate UIP pattern is used when HRCT demonstrates some features of fibrosis but does not meet the UIP or probable UIP criteria. This includes patients that show very limited sub-pleural ground-glass opacification or reticulation. Finally, there may be cases where IPF is suspected at the clinical level, however the HRCT pattern suggests an alternative diagnosis.[9]

Following the use of HRCT, surgical lung biopsy (SLB) or video-assisted thoracoscopic surgery may be pursued in order to secure a diagnosis of IPF.[9] The histopathology features that represent the UIP pattern are associated with the appearance of patchy fibrosis that is implicated in the remodelling of lung architecture, often resulting in honeycombing, and variations in the degree of affected areas in the lung parenchyma. The subpleural parenchyma is typically affected the most severely.[16] Inflammation is generally modest and consists in the presence of a lymphocytic and plasma cell infiltrate stemming from hyperplasia of type II pneumocytes and bronchial epithelium.[9] In addition, fibrotic zones, known as fibrotic foci, can be distinguished by the heavy amount of collagen as a result of proliferating fibroblasts and myofibroblasts. Honeycombing can be seen under the microscope as a result of cystic fibrotic airspaces that are lines by bronchiolar epithelium and filled with mucus and inflammatory cells.[16] As a result of these histopathologic features, a definitive diagnosis of the UIP pattern can be made when all of the above features are found in addition to the honeycombing pattern seen in HRCT.[9] In order to properly diagnose IPF, HRCT is used to define a UIP pattern and can be supplemented with histopathological features in order to confirm UIP and probable UIP as IPF or as an alternative disease.[9]

1.1.3 IPF: Current treatments

The current therapeutic strategy for the treatment of IPF revolves around nonpharmacologic and pharmacologic approaches with the intent of slowing down disease progression and reducing symptoms.[17] It is crucial to begin preventative care, rehabilitation, and symptom-based treatment as early as possible in order to counter a decline in quality of life.[18] Many patients suffer from inactivity due to shortness of breath which leads to loss of muscle mass and increasing fatigue. Therefore, pulmonary rehabilitation is a recommended approach to help reduce the threshold of dyspnea and improve the functional status of patients through symptom alleviation.[19] The use of lung transplantation is an established therapeutic option in the treatment of chronic lung disease, however patients suffering from IPF only represent 35% of transplant recipients.[20] For patients that do undergo the procedure, the risk of death has been shown to be lowered by 75% and a more favourable long-term survival has been reported.[21] The landscape of pharmacological treatment for IPF remains relatively limited with only two anti-fibrotic molecules currently approved.[22] The use of pirfenidone (Esbriet®) and nintedanib (Ofev® and Vargatef®) in IPF patients with mild or moderate impairment in forced vital capacity (FVC) has been shown to reduce FVC decline, reduce acute exacerbations, and preserve health-related quality of life.[17,23] Therefore, most IPF patients may benefit from improved lung function using one of these two therapeutic options.

Pirfenidone is a small molecule that has both antifibrotic as well as anti-inflammatory properties.[24] The exact mechanism of action remains unknown, however following five clinical trials pirfenidone is a safe option in reducing FVC decline.[17,24] On the other hand, nintedanib is a potent small molecule receptor tyrosine kinase (RTK) inhibitor that works by targeting platelet-derived growth factor (PDGF) receptor alpha and beta, fibroblast growth factor receptors (FGFR) and vascular endothelial growth factor receptors (VEGFR).[25] Nintedanib competitively binds at the ATP binding pocket site of these receptors, leading to inhibition of intracellular signalling.[25]

Following three international clinical trials, nintedanib has provided an additional option in reducing FVC, reducing exacerbations and preserving a health-related quality of life compared to placebo.[17] Ultimately, IPF remains a fatal disease, and more research is crucial to help find better treatment options to help patients suffering from the disease.

1.2 Pathogenesis of IPF

1.2.1 Overview of IPF pathogenesis, risk factors

Over the last few years, several important milestones have been reached in the understanding of the IPF pathogenesis. Various environmental, microbial, and genetic factors have been proposed to play important roles in IPF pathobiology, despite the cause remaining elusive.[26] Several risk factors are thought to contribute to the development of the fibrotic process by driving repeated micro-injury to the alveolar epithelium. Subsequently, lung cells develop aberrant behaviours in the repair process leading to the development and sustainment of the fibrotic process.[3] The following section will take an in-depth look at the current state of knowledge regarding risk factors and the abnormal processes taking place in lung parenchyma.

There are several different environmental risk factors that may be involved in the pathogenesis of IPF. Cigarette smoking as well as other inhaled pollutants such as metal and wood dusts have been associated with the risk of developing IPF, including the familial form of pulmonary fibrosis.[26] Viral, fungal, and bacterial microbial agents have been linked as potential risk factors for the disease.[27] Recent research has suggested an imbalance in the bacterial communities observed in IPF lungs versus healthy lungs, which may even offer a future prognostic biomarker.[28] In an analysis of bronchoalveolar lavage (BAL) from patients with IPF, viral infection such as with Epstein-Bar-virus, cytomegalovirus, hepatitis C and human herpesvirus-8 were found.[27]

IPF susceptibility appears to arise from several abnormal genetic features like gene variants and transcriptional changes that may play a role in the loss of epithelial integrity of the lung.[3] In studies on patients with the familial form of the disease, genetic variants have been linked with maintenance of telomere length and surfactant dysfunction. These same variants have also been recognized in those with the sporadic form of the disease.[29] Moreover, two large genome wide association studies (GWAS) have reported common genetic variants that are important in telomere biology, host defence and cellular barrier function. These genetic mutations play a crucial role in destabilizing the alveolar epithelium.[30,31]

1.2.2 Cells, cytokines and growth factors

Currently, IPF is widely considered to be an epithelium-driven disease due to the combination of ageing and recurrent microinjuries that lead to impaired regeneration of epithelial tissue. In addition, there is an imbalance between profibrotic and antifibrotic mediators.[32] The prevailing thought is that fibrosis likely evolves over many years prior to diagnosis in patients, where there is a modified lung structure, epithelial cell hyperplasia, dense fibrosis and abnormal proliferation of mesenchymal cells.[3] The alveolar epithelium is comprised of two main cell types known as type I alveolar epithelial cells (type I AECs) and type II alveolar epithelial cells (type II AECs).[33] Type I AECs are complex branched cells that are highly attenuated and comparatively devoid of organelles as they make up the gas exchange surface in the alveolus. Type II AECs respond to damaged type I AECs by acting as the progenitor cell for both types of alveolar epithelial cells. Type II AECs also store and release surfactant into the alveolar hypophase in order to stabilize the gas exchange process.[33] Therefore, under normal conditions, type I AECs that are lost due to injury are replaced by proliferating and differentiating type II AEC stem cells that restore the integrity of the alveolar membrane. Several mechanisms are involved such as the coagulation cascade, new vessel formation, fibroblast activation and migration, collagen synthesis and proper alignment.[34]

Notable chemokines involved in these processes are TGFβ1, PDGF, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).[35]

TGFβ1 is one of the most important mediators in the pathogenesis of IPF. The levels of TGFβ1 are increased in the lungs of patients with IPF.[36] Type II AECs produce TGFβ1 through the actin/myosin-mediated cytoskeletal contraction of the αvβ6 integrin.[37] The αvβ6 integrin/ TGFβ1 pathway is a well-conserved system that plays an important role in the detection of injurious stimuli. Under normal circumstances, inactive TGFβ1 is bound to the latency associated peptide (LAP) which is crosslinked by disulfide bonds to latent TGFβ1 binding protein (LTBP) and covalently bound to the ECM.[38] This is known as the TGFβ1 large latent complex (LLC) and acts to keep TGFβ1 in an inactive state (Figure 1.1).[38] In lung fibrosis, type II AECs express increased levels of αvβ6 integrin, which has the ability to bind to LAP and release TGFβ1.[39] Alongside mediators that induce contraction of epithelial cells such as thrombin, sphingosine 1-phosphate and lysophosphatidic acid, type II AECs pull on the TGFβ1 that is bound to the αvβ6 integrin thereby activating the chemokine. Another source of TGFβ1 is the unfolded protein response (UPR) that arises from the dysfunction of type II AECs.[36] As a result of protein over-expression and endoplasmic reticulum (ER) stress, the UPR is activated to re-establish normality.[40] Amongst the several consequences of the UPR, the ability to stimulate the production of profibrotic mediators such as TGFβ1 and PDGF is of high importance in IPF pathogenesis.[3] Ultimately TGFβ1 is involved in a plethora of profibrotic responses including epithelial cell apoptosis; epithelial mesenchymal transition (EMT); epithelial cell migration; production of other profibrotic mediators; circulating fibrocyte recruitment; and fibroblast activation, proliferation and

transformation into myofibroblasts.[35] These processes will be discussed further below as they are instrumental in the development of IPF.

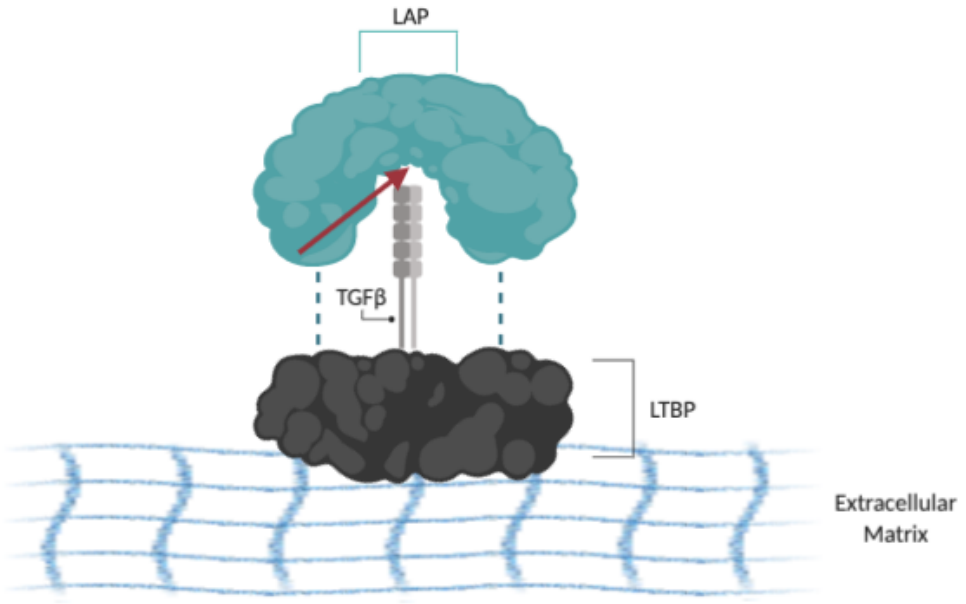


Figure 1.1: The TGFβ1 large latent complex (LLC). The LLC is made up of the LAP (blue), TGFβ1 (grey) and LTBP (black). The LAP is linked to the LTBP by disulfide linkages (dotted lines). As a result of contraction mediated by αvβ6 integrin, LAP and TGFβ1 can be proteolytically separated (red arrow). Dissociation of LAP from TGFβ1 allows for endogenous release of TGFβ1. Information adapted from Annes et al. [38]

In addition to AECs, mesenchymal cells are crucial to the pathogenesis of IPF. In particular, fibroblasts and myofibroblasts are recruited, activated and induced to differentiate and proliferate as a result of an abnormal biochemical environment from activated epithelial cells.[41] The initial trigger for recruitment of these mesenchymal cells is still unknown, although the current literature suggests that pulmonary fibroblasts, circulating fibrocytes and myofibroblasts are all involved.[41] Tissue mesenchymal cells known as fibroblasts are found in connective tissue where they produce ECM proteins such as collagen in order to establish proper structural framework in tissues and during the wound healing repair processes.[42] Throughout the course of IPF, fibroblasts are exposed

to secreted profibrotic mediators from surrounding alveolar epithelial cells and activated fibroblasts; this leads to ECM production and the differentiation of fibroblasts to myofibroblasts. TGFβ1 is essential to the differentiation process alongside other mediators such as PDGF.[3] Chronic exposure to these mediators leads to the classic pathologic phenotype of fibroblasts described in the lungs of IPF patients. This is due to the fact that myofibroblasts, compared to resident lung fibroblasts, secrete excessive amounts of ECM proteins, such as type I collagen and fibronectin.[43] The ECM is very dense and myofibroblasts tend to persist longer than fibroblasts in damaged tissue. Similar to smooth muscle cells, myofibroblasts possess contractile properties due to their expression of α-SMA. Myofibroblasts undergo irreversible contraction that leads to collagen remodeling, changes in spatial organization of collagen fibrils, and imparts mechanical stress that results in a stiffening ECM.[43] An important regulating factor of myofibroblasts is the mechanical composition of the ECM. As a result of dense and stiff ECM, myofibroblasts actually increase their synthetic activity, creating a positive feed-back loop that ultimately results in the invasive nature of this cell type.[3]

The increase in fibroblasts and myofibroblasts can also be linked with EMT, a process in which epithelial cells undergo phenotypic change resulting in molecular and physiologic features of mesenchymal cells.[44] This process is initiated following activation by specific growth factors, the most common being TGFβ1. Genes commonly expressed by mesenchymal cells such as α-SMA are upregulated whereas genes associated with epithelial cells (*e.g.* E-cadherin) are downregulated, resulting in decreased polarity and tight junctions, both being characteristics of epithelial cells.[45] The evidence for EMT occurring in patients with IPF can be seen in data that shows AEC II markers (*e.g*. prosurfactant protein B) colocalized with α-SMA in the majority of IPF lungs.[46] Although there is evidence for EMT in the lungs of IPF patients, it is still unresolved to what extent

the contribution of these mesenchymal changes is having in the pathogenesis of the disease.[36] EMT can also be associated with endothelial cells undergoing mesenchymal transition and is an emerging concept in the pathology of IPF.[47] As a result of damage to the alveolar epithelium and alteration of the basement membrane in IPF, alveolar vessel disruption and vascular permeability may occur. Under normal circumstances, endothelial progenitor cells (EPC) respond by inducing new vessel formation and endothelial cell proliferation. However, patients with IPF may have decreased EPCs. The consequences of this reduction is dysfunction of alveolar-capillary barrier and a profibrotic response with augmented levels of VEGF, which induces endothelial cells to undergo mesenchymal transition and aids in the differentiation of fibroblasts to myofibroblasts.[47]

Fibrocytes represent a class of mesenchymal cells that are derived from the bone marrow and can be found in the circulation as well as tissues. Fibrocytes have been implicated in IPF pathogenesis as they contribute directly to the deposition of collagen and other ECM proteins by differentiating into fibroblasts or myofibroblasts or by producing cytokines that promote collagen accumulation.[48] Not only are fibrocytes increased in both the circulation and lung tissue of patients with IPF, but patients with fibrocytes >5% of their total circulating leukocytes, where 1% is considered normal, have a worse prognosis than those who have <5%, suggesting their contribution to lung fibrosis.[36,49] The recruitment of circulating fibrocytes stems from the alveolar epithelium expressing chemokine ligands CXCL12 and CCL2 that bind to receptors found on fibrocytes. Levels of CXCL12 and CCL2 are increased in the circulation and in lung parenchyma of IPF patients. These findings suggest that the recruitment and differentiation of fibrocytes likely play a role in the pathogenesis of IPF.[48]

1.2.3 Metabolic changes in IPF, glycolytic shift

A shift in metabolism has been shown to occur in a variety of different diseases such as Alzheimer's, cancer and diabetes. Metabolic changes arise from altered mitochondrial function typically associated with an increase in glycolysis.[50] While differentiating and proliferating cells have been associated with IPF pathogenesis, emerging evidence for metabolic dysregulation have been identified within cells of IPF lungs.[51] These metabolic changes may play a role on lung cell function, differentiation and activation of fibrotic responses. Metabolic alterations can result in the activation of TGFβ1, predisposing the lungs to a fibrotic environment.[52] It should be noted that alterations in metabolism can occur through normal ageing and thus may explain why the frequency of IPF increases with age. This would suggest that metabolic changes could lead to increased susceptibility of IPF.[51]

Studies investigating metabolic activity in fibrotic lung tissue have analyzed the uptake of ^{18}F-fluorodeoxyglucose (FDG), a glucose analog measured by positron emission tomography (PET) scan, and discovered an increase in FDG uptake which suggests increased glucose metabolism.[51,53] Interestingly, in a study of 36 patients with IPF and ILD, all showed areas of increased FDG metabolism- greater than twice the values observed in normal lung tissue.[54] Furthermore, the areas of abnormal parenchymal architecture corresponded with increased uptake. In areas of the lung that presented the characteristic honeycombing pattern, FDG uptake was the most intense. PET scan images revealed an increase in glucose transporter-1 mediated metabolism. This provides a link between increased glucose metabolism and areas believed to be irreversibly affected by pulmonary fibrosis.[54] Although the explanation for raised FDG still remains unclear, it is important to note that fibroblasts, central to IPF pathology, are known to express the glucose transporter-1 (GLUT-1).[51] This could mean that increased FDG uptake, particularly in sites of honeycombing, could be a reflection of increased fibroblast metabolism.

Glucose metabolism begins with its import via glucose transporters into the cytoplasm of cells, where it is converted to pyruvate through a series of enzymatic conversions known as glycolysis (Figure 1.2).[55,56] There are three main rate-limiting reactions within the glycolytic pathway that are catalyzed by three separate enzymes: hexokinase II (HKII), phosphofructokinase 1 (PFK1), and pyruvate kinase (PK). The reactions catalyzed by these enzymes are all irreversible and often subject to regulation. Under circumstances where there is sufficient oxygen present (aerobic conditions), pyruvate is then shuttled into the tricarboxylic acid (TCA) cycle and processed into approximately 30-38 ATP via oxidative phosphorylation. This process is generally how cells produce energy in the most efficient manner.[55] Under conditions of low oxygen (anaerobic conditions), pyruvate is simply broken down into lactate in the cytosol and then secreted from cells.[52] Metabolism that results in lactate production is commonly referred to as 'glycolysis.' An increase in glycolysis can also occur in the presence of normal oxygen conditions, known as the "Warburg effect", and most notably can be seen in tumour cells.[57] An analysis of human lung myofibroblasts treated with TGFβ1 conducted by Xie *et al.* demonstrated an increase in lactate production and extracellular acidification indicating increased glycolysis. In addition, key glycolytic enzymes, PFK1 and HKII were significantly increased, suggesting that augmented glycolysis in these cells stems from enzymes that irreversibly catalyse this process. The use of a glycolytic inhibitor also showed that a reduction in glycolysis reduced myofibroblast differentiation following TGFβ1 treatment.[55] These results suggest that a glycolytic shift may be an early event in the differentiation of lung myofibroblasts and an important step in the development of fibrosis.

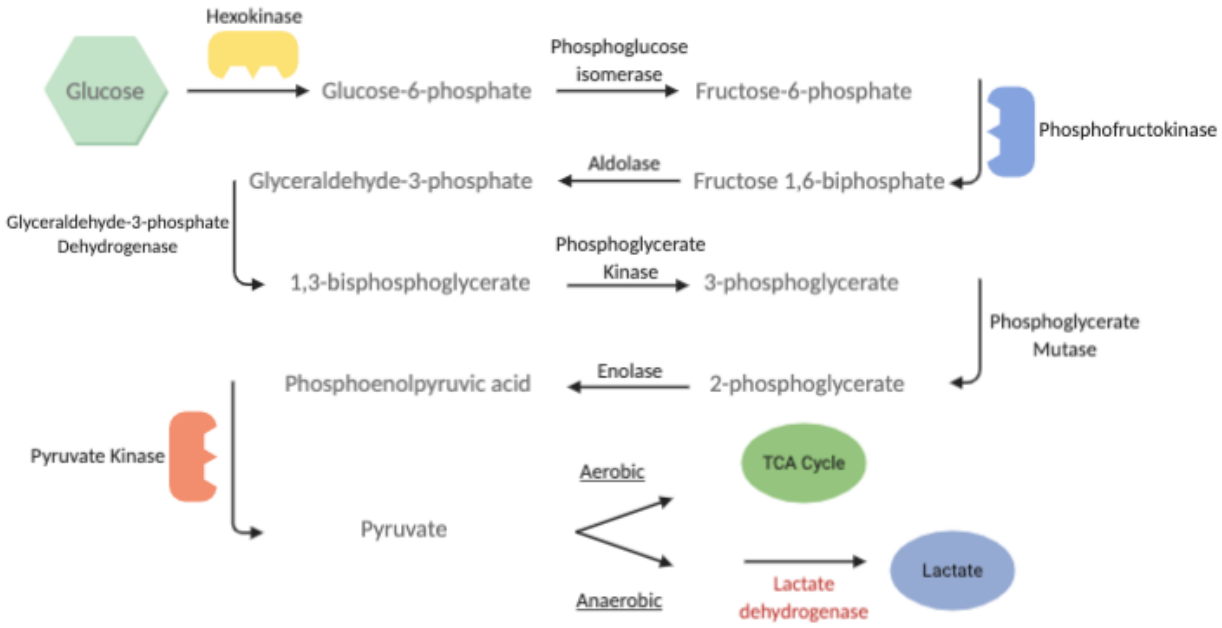


Figure 1.2: A schematic representation of the classical pathway of glycolysis demonstrated under aerobic and anaerobic conditions. Hexokinase, phosphofructokinase and pyruvate kinase represent the three most important enzymes that catalyze the formation of pyruvate from glucose. Under aerobic conditions pyruvate is shuttled to the TCA cycle while under anaerobic conditions pyruvate is converted to lactate by lactate dehydrogenase (red). Information adapted from Fan et al. [56]

Metabolic analysis of lung tissue from IPF patients has revealed that the end product of glycolysis lactic acid- is significantly elevated in lung tissue compared with healthy control subjects.[52] When primary human lung fibroblasts were treated with TGFβ1, higher concentrations of lactic acid were found in the supernatant of treated cells versus untreated cells. Furthermore, lactate dehydrogenase (LDH), the enzyme responsible for catalyzing the formation of lactic acid, is elevated in both fibroblasts from lung tissue of IPF patients as well as in primary human lung fibroblasts treated with TGFβ1.[52] Upon closer inspection, the more prominent expression of LDH was found in epithelial cells and fibroblasts that were in and surrounding fibroblastic foci which corresponded with areas of increased lactic acid. Interestingly, the production of lactic acid leads to a drop in pH

which can subsequently activate latent TGFβ1. The addition of lactic acid at biologically-relevant concentrations induced myofibroblast differentiation when latent TGFβ1 was present.[52] These results further support the notion that an increase in glycolysis leads to a pro-fibrotic environment that induces differentiation.

1.2.4 Hypoxic Microenvironment and hypoxia inducible factor 1-alpha (HIF-1α)

Hypoxia plays an important role in normal wound healing. As a result of compromised vascular perfusion from tissue injury, hypoxia can induce specific gene expression to re-establish oxygen delivery to the tissue.[58] Hypoxia induces a signalling cascade through HIF-1 which activates pathways that can promote repair to restore tissue function.[59] In the case of chronic tissue injury, hypoxia may actually drive fibrosis, ultimately leading to scarring and loss of organ function.[58]

HIF-1 is an αβ heterodimer, where the β subunit is constitutively nuclear and the α subunit is inducible under hypoxic conditions. Under normal physiological oxygen conditions, prolyl hydroxylases, which are dependent upon molecular oxygen to maintain stability at the protein level, direct HIF-1α to be poly-ubiquinated and subsequently destroyed by the proteasome.[59] In contrast, under hypoxic conditions prolyl hydroxylases are inactivated, allowing HIF-1α to escape degradation and migrate into the cell nucleus where it can bind to HIF-1β and induce transcription of approximately 70 target genes.[59,60] These target genes are involved in several process, mainly to promote glycolysis, angiogenesis, erythropoiesis and regulate vasomotion. In the context of fibrosis, HIF-1α has been shown to increase TGFβ1 downstream signalling thereby inducing ECM production. TGFβ1 signalling proteins, SMAD 2/3, p-SMAD2/3, and SMAD-4, have been observed to be up-regulated in the presence of hypoxia via HIF-1α.[61] SMAD-4 binds to phosphorylated SMAD2/3 to form the SMAD2/3/4 complex which translocates to the nucleus to

regulate TGFβ1 target genes, including collagen. Thus, HIF-1α plays an important role in the activation of TGFβ1/SMAD signalling and the promotion of pro-fibrotic processes.[61,62] In the context of metabolic changes, HIF-1 has been shown to increase enzymes and transporters important in glycolysis such as HKII and PFK while also increasing the activity of LDH and decreasing enzymes that direct metabolism towards oxidative phosphorylation.[63] Analysis of human lung tissue from IPF patients revealed a marked increase in HIF-1α compared with healthy lung samples.[64] In the context of human lung fibroblasts (HLFs), TGFβ1 activates HIF-1α and the over-expression of HIF-1α induces LDH, leading to increased myofibroblast differentiation *in vitro*.[52] Inhibition of HIF-1α reduces fibroblast differentiation to myofibroblasts and LDH levels following TGFβ1 treatment. Therefore, it is plausible that there exists a feed-forward loop between TGFβ1, HIF-1α and LDH, such that increased lactic acid can activate TGFβ1, which in turn increases HIF-1α and LDH expression to generate additional lactic acid.[52] Thus, a glycolytic shift may play a role in the pathogenesis of IPF.

1.2.5 Molecular regulation

Understanding further the molecular regulation of these pathogenic mechanisms may lead to better approaches and treatments for IPF. Epigenetic alterations such as DNA methylation have been observed in patients with IPF.[3] Another manner of regulation is at the level of microRNAs (miRNA) which can silence genes through the degradation of target mRNA or inhibiting translation.[65] For example, miR-21 activates TGFβ1 signalling by targeting Smad7, a specific gene of the TGFβ1 pathway which leads to the activation of the profibrotic cytokine and promotes differentiation of fibroblasts.[66] The half-life and translation of mRNA may also be influenced by RNA-binding proteins (RBPs).[67] For example, Insulin-like growth factor binding protein (IGFBP-5) promotes fibrosis by increasing ECM and pro-fibrotic genes in primary HLFs.[68] An RBP that

has been implicated in the regulation of several genes relevant to IPF is the human antigen R (HuR).[67] Not only has HuR been associated with profibrotic genes such as *COL1A1* and *ACTA2,* but HuR also increases the translation of HIF-1α, which has implications in both differentiation and metabolic shift seen in fibrotic cells.[69,70]

1.3 Human antigen R (HuR)

1.3.1 Overview and structure of HuR

Eukaryotic gene expression is regulated at transcriptional and post-transcriptional levels before it is translated into protein.[71] Post-transcriptional regulation occurs at the level of processing, transport, stability, and translation, all of which can be carried out by RBPs and noncoding RNAs (ncRNA). These trans-acting factors can bind to cis elements present in mRNA, allowing them to regulate stability and translation.[67] One of the best described RBP was first described in *Drosophila melanogaster* as *elav* (embryonic lethal abnormal vision) and is known today in mammalian cells as HuR which is ubiquitously expressed in comparison to the primarily neuronal proteins HuB, HuC and HuD.[72] The HuR protein contains three highly conserved RNA recognition motifs (RRMs), where RRM1 and RRM2 show high affinity towards U and AU rich elements (AREs), mainly in the 3' untranslated region (UTR) of target mRNA.[73] The interaction of HuR and poly(A)tails of target mRNA is carried out by RRM3 and is thought to be involved during the assembly of HuR oligomers.[74] In between RRM2 and RRM3 is the HuR nucleocytoplasmic shuttling sequence (HNS) allowing for nuclear/cytoplasmic localization of HuR.[75] The AREs that HuR interacts with consist of several adenine and uridine residues present in approximately 4800 mRNA, including growth factors, cytokines and lymphokines. Several of these mRNAs play important roles in cell growth, angiogenesis, and metastasis.[67,76]

1.3.2 HuR function and regulation

Central to the function of HuR is its ability to translocate from the nucleus to the cytoplasm where it can bind target mRNA in both locations. In general, HuR binds to mRNA in the nucleus and shuttles them to the cytoplasm, providing protection from degradation machinery.[71] External stress such as UV and heat shock increase cytoplasmic translocation of HuR. As a result of UV exposure, for example, HuR translocates and stabilizes p21 mRNA, which inhibits the growth of damaged cells.[77] Post-translational modification of HuR by phosphorylation affects localization and interaction with target mRNA.[78] Modifications that surround the RRMs of HuR alter binding with mRNA whereas changes to the HNS alter its localization.[67] Levels of HuR protein are regulated through several different mechanisms such as auto-regulation, downregulation by miRNAs, ubiquitination and cleavage.[79-82] Similar to other RBPs, HuR can bind its own mRNA to increase stability and cytoplasmic transport.[83] miR-519 and miR-125a are two miRNA that can associate with *ELAVL1* (embryonic lethal abnormal vision-like protein 1) mRNA and promote its degradation.[80,84] HuR abundance, integrity, and post-translational modifications involve several tightly controlled processes that play a pivotal role in HuR binding and function.[67]

1.3.3 HuR in fibrosis

HuR is involved in several diseases ranging from cancer, inflammation, cardiovascular disease and fibrosis.[69,85-87] Early reports of HuR being upregulated in brain and colon tumors showed that it increased the expression of cyclooxygenase-2 (COX-2), VEGF, TGFβ, IL-8 and many other cancer-associated proteins.[85] Subsequently, HuR was found to be elevated in the vast majority of malignancies where it is thought to play a role in tumor development. HuR controls the expression of mRNA whose protein products are important in proliferation, cell survival, angiogenesis, immune evasion and metastasis.[88] Many target genes for HuR include proteins involved in the cell cycle progression and cell division such as c-Fos and epithelial growth factor (EGF). Moreover, HuR suppresses proteins that have growth inhibitory roles which then allows for proliferation in a

variety of pro-growth pathways.[67] In promoting angiogenesis, HuR can bind, stabilize and promote translation of VEGF and HIF-1α.[70,89] Cytoplasmic HuR levels are associated with tumour stage progression, resulting in poor prognosis for patients with breast cancer and colon cancer carcinomas.[90,91] HuR has also been implicated in promoting inflammation and inflammatory diseases through its ability to upregulate pro-inflammatory proteins and cytokines while also being inhibited by anti-inflammatory factors.[85] The three most prominent inflammatory cytokines that HuR interacts with are (tumor necrosis factor alpha) TNF-α, TGFβ and IL-6, by stabilizing and promoting their expression in a variety of cell types, including fibroblasts and macrophages.[92]

In the context of fibrosis, HuR may contribute to disease pathogenesis in the liver and heart. In both cases, HuR is elevated at the protein level and translocates to the cytoplasm in response to PDGF, TGFβ and angiotensin II .[69,93] This translocation increases TGFβ activation that coincided with increased collagen synthesis and α-SMA production. Inhibiting HuR had a negative effect on the ability of cells to differentiate in response to PDGF and TGFβ.[69] While evidence continues to emerge in the role HuR plays in liver and cardiac fibrosis, research on HuR in pulmonary fibrosis is still completely unknown.

Chapter 2 HYPOTHEISIS AND AIMS

2.1 Hypothesis:

HuR regulates hypoxia-induced metabolic reprogramming and myofibroblast differentiation in human lung fibroblasts.

2.2 Aims:

Aim 1: Assess the effect of hypoxia on HuR expression and cellular localization in human lung fibroblasts.

Aim 2: Investigate the effect of hypoxia on metabolic reprogramming and myofibroblast differentiation in human lung fibroblasts.

Aim 3: Investigate the role of HuR in hypoxia-induced metabolic reprogramming and myofibroblast differentiation in human lung fibroblasts.

Chapter 3 MATERIALS AND METHODS

3.1 Reagents

The name and company where reagents were purchased from will be described throughout the methodology section.

3.2 Subject characteristics of HLFs

HLFs used in this study were derived from lung tissue obtained through lung resection surgery in subjects undergoing the procedure at McMaster University.[94] The research ethics board of St Joseph's Healthcare Hamilton approved this study and written consent was obtained from each subject. This study was conducted on HLFs from three different subjects with no smoking history and or other known risk factors (*e.g*. radiation therapy) for lung fibrosis. Clinical characteristics of patients are shown below in Table 1.

Table 1. Subject clinical characteristics

	Normal
No. of subjects	3
Age	68±15.6
Gender (M/F)	1/2
Years of smoking	0.0
FEV_1(%)	94.3±11.9
FVC (%)	93.3±5.8
FEV_1/FVC (%)	79.18±9.7

3.3 Cell Culture

HLFs were derived by explant procedure from cancer-free lung tissue.[94] Cells were cultured in Gibco Minimum Essential Media (MEM) (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) in addition to gentamycin (WISENT Inc, Canada) and Antibiotic-Antimycotic (WISENT Inc, Canada). HLFs from three separate non-smoker individuals were used for all experiments with cell passage number being between passage 6 and 9.

3.4 Cell Treatments

HLFs were cultured under four separate conditions. Under normoxia, HLFs were incubated in humidified chambers at 37^{o}C and were exposed to 21 % O_2 and 5% CO_2. Under hypoxia, HLFs were incubated in humidified culture chambers at 37^{o}C and were exposed to 1% O_2 and 5% CO_2. This was accomplished by using the Xvivo System Model X3 hypoxia incubator (BioSpherix Ltd, USA) which was attached to three separate gas tanks containing N_2, air, and CO_2. 1% O_2 was chosen according to previous dose response experiments inducing HIF-1α without causing cytotoxicity (data not shown). Under both normoxia and hypoxia conditions, HLFs were either left untreated or treated with human recombinant TGFβ1 (R&D Systems, USA), at a concentration of 5ng/ml based on previous dose response experiments (data not shown).

3.5 Immunofluorescence

HLFs were treated under normoxia, hypoxia, TGFβ or hypoxia plus TGFβ1 for 4 hours, followed by fixation with paraformaldehyde for 15 minutes and permeabilized for 30 minutes in phosphate-

buffered saline (PBS) containing 0.5% Triton. Once HLFs were incubated with blocking buffer (Dako, USA) for 1 hour at room temperature, cells were then incubated in a 1:300 dilution of anti-HuR antibody (Santa Cruz, USA) in antibody diluent (Dako, USA) or with antibody diluent only for 2 hours at room temperature. After PBS washes, cells were then incubated in a 1:1000 dilution of secondary antibody Alexa fluor 555 (Invitrogen, USA). Then HLFs were washed with PBS and nuclei were stained in a 1:1000 dilution of 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate (Hoechst 33342) (Thermo Fisher, USA). Microscopy images were acquired through the Zeiss LSM 780 confocal microscope (Zeiss, Germany). ICY software was used for bioimage analysis to quantify HuR cytoplasmic translocation.

3.6 Quantitative RT-PCR (qPCR)

HLFs were cultured with serum free MEM for 18 hours prior to treatment under normoxia, hypoxia, TGFβ1 or hypoxia plus TGFβ1 for 24 and 48 hours. Then, total RNA was isolated with Trizol in accordance with the manufacturer's instructions. The quantification of RNA was done by using a Nanodrop 1000 spectrophotometer infinite M200 pro (Tecan, Switzerland). Reverse transcription of RNA to cDNA was accomplished using iScript Reverse Transcription Supermix (Bio-Rad laboratories, Canada) and mRNA levels of *ELAVL1, ACTA2, FN1, COL1A1, LDHA* and *HK2* were analyzed using gene specific primers (Table 2). Quantitative real-time (reverse transcription) polymerase chain reaction (qPCR), was carried out by combining 1µl cDNA and 0.5 µM primers with SsoFast EvaGreen (Bio-rad laboratories, Canada) and amplification was performed using a CFX96 Real-Time PCR Detection System (Bio-rad laboratories, Canada). The procedure of thermal cycling was initiated at 95ºC for 3 minutes and was followed by 39 cycles of

denaturation at 95°C for 10 seconds and annealing at 59°C for 5 seconds. Genomic RNA expression was analyzed using the ΔΔCt method, and results are presented as fold-change normalized to the housekeeping gene (*S9*).

Table 2: primer sequences used for qRT-PCR analysis

Gene	Forward Primer Sequence	Reverse Primer Sequence
ELAVL1	AACGCCTCCTCCGGCTGGTGC	GCGGTAGCCGTTCAGGCTGGC
COL1A1	CAGACTGGCAACCTCAAGAA	CAGTGACGCTGTAGGTGAAG
ACTA2	GACCGAATGCAGAAGGAGAT	CACCGATCCAGACAGAGTATTT
FN1	CTGAGACCACCATCACCATTAG	GATGGTTCTCTGGATTGGAGTC
S9	CAGCTTCATCTTGCCCTCA	CTGCTGACGCTTGATGAGAA
LDHA	GGAGATTCCAGTGTGCCTGT	CGTAAAGACCCTCTCAACCACC
HK2	GGGCGGATGTGTATCAAT	GTGAGCCCATGTCAATCT

3.7 Western Blot

HLFs cultured with serum free MEM for 18 hours followed by exposure to normoxia, hypoxia, TGFβ or hypoxia plus TGFβ1 for 24, 48, and 72 hours once the cells reached 70-75% confluence. Total cellular protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific, USA) in conjunction with Protease Inhibitor Cocktail (Roche, USA). Following extraction, protein concentration was measured by the bicinchoninic acid (BCA) protein kit (Thermo Fisher Scientific, USA). Protein lysate at a concentration of 2 or 20 μg was electrophoresed on 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels and transferred onto Immuno-blot PVDF membranes (Bio-Rad Laboratories, USA). After transfer, the membrane was blocked with a blocking solution of 5% w/v non-fat dry milk in 1x PBS/0.1% Tween-20 for one

hour at room temperature. Antibodies were applied to membranes for 1 hour or overnight. The following is a list of the antibodies used: anti-HuR (1:2000; Santa Cruz, USA), anti-α-SMA (1:5000; Sigma-aldrich, USA), anti-Col1A1 (1:200; Santa Cruz, USA), anti-HIF-1α (1:1000; abcam, USA), anti-FN (1:200; Santa Cruz, USA) and anti-Tubulin (1:50000; Sigma-aldrich, USA). After the application of the primary antibody, the secondary antibodies used were anti-rabbit IgG, horseradish peroxidase (HRP) linked (1:10000; Cell Signalling Technologies, USA) and HRP conjugated anti-mouse IgG (1:10000; Cell Signalling Technologies, USA). Membrane visualization was performed by using Clarity western enhanced chemiluminescence (ECL) substrate (Bio-Rad Laboratories, Canada) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA). Detection of protein bands was done by the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Canada). Densitometric analysis was analyzed through Image Lab Software Version 5 (Bio-Rad Laboratories, Canada) and protein expression was normalized to tubulin and presented as fold change compared to the normoxia condition.

3.8 Metabolic profiling by Proton Nuclear Magnetic Resonance (^{1}H NMR)

HLFs were treated under normoxia, hypoxia, TGFβ1 or hypoxia plus TGFβ1 for 24, 48, and 72 hours after being cultured with serum-free MEM media for 18 hours. Cell supernatant was collected and supplemented with 10% D_2O for shift lock and 1mM total soluble protein (TSP) standard (Sigma Aldrich, USA). The ^{1}H spectra was obtained using a 500-MHz Bruker machine using a pre-saturation method following 128 scans. Spectra analysis was performed using ACD Labs software (Advanced Chemistry Development UK Ltd, UK) and integrals for peaks at 5.2 ppm (glucose) and 1.3 ppm (lactate) were quantified in relation to the standard internal TSP peak at 0.0 ppm.

3.9 HuR-siRNA knockdown

HLFs were seeded at 10 x10^4 cells/cm^2 and one day later were transfected with 60 nM of small-interfering RNA (siRNA) targeting HuR (Santa Cruz, USA) or non-targeting control siRNA (Santa Cruz, USA) in accordance with manufacturer's instructions. The transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, USA) in accordance with manufacturers directions. After one hour, 10% MEM medium was added onto the cells. Twenty-four hours later, fibroblasts were treated with serum-free MEM medium for 18 hours, followed by treatment under normoxia, hypoxia, TGFβ1 or hypoxia plus TGFβ1 for 72 hours. Confirmation of siHuR knock-down was examined by western blot 114 hours after transfection.

3.10 Statistical Analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test to assess the differences between normoxia, hypoxia, TGFβ1, and hypoxia plus TGFβ1 at the various time-points. This done using GraphPad Prism 6 (v.602; GraphPad Software Inc, USA). Results are presented as mean ± standard error of the mean (SEM) of the fold changes compared to normoxia. Statistical significance was considered in all cases which had a p value < 0.05.

Chapter 4 CONTRIBUTION OF CO-AUTHORS

This thesis is founded on original work and has been prepared as a manuscript for the purpose of peer-review and submission.

- Human lung fibroblasts used in this project were generated by lung tissue provided by Dr. Parameswaran Nair in the Department of Medicine at McMaster University.
- Dr. David H. Eidelman assisted in experimental design and project management.
- Dr. Ilan Azuelos provided intellectual support and guidance as well as experimental design, project management and thesis editing.
- Dr. Carolyn Baglole provided intellectual support and guidance as well as experimental design, project management and thesis editing.

Chapter 5 RESULTS

5.1 Fibroblast differentiation into myofibroblasts is reduced in response to hypoxia and TGFβ1.

Our first question focused on the effect of hypoxia on the differentiation of fibroblasts to myofibroblast, a process central to IPF pathogenesis, as myofibroblasts potently increase the production of the ECM with stiffens the lungs.[41] Increased HIF-1α in the lung tissue of IPF patients has also implicated hypoxia as contributing factor in disease pathogenesis.[64] To address whether hypoxia contributes to myofibroblast differentiation, we induced differentiation with TGFβ1 to compare the effects with and without hypoxia.[52] To assess differentiation of HLFs, we analyzed the expression of α-SMA (*ACTA2*), a marker of myofibroblasts, as well as key ECM components including collagen 1 (*COL1A1*) and fibronectin 1 (*FN1*) when cells were exposed to hypoxia (1% O_2), TGFβ1 (5ng/ml) and hypoxia with TGFβ1 at 24-, 48- and 72-hours by qPCR and western blot. HLFs treated under normoxia (21% O_2) were used as a negative control. At the mRNA level, TGFβ1 significantly increased *ACTA2* and *FN1* at 48 hours (Figure 5.1A and 5.1C). Hypoxia alone had negligible effects on mRNA levels, however, in combination with TGFβ1 there was a significant increase in *ACTA2* at 24- and 48-hours as well as a significant induction of *FN1* at 48 hours. (Figure 5.1A and 5.1C). We did not find significant changes in *COL1A1* mRNA with any treatment, although there was an increasing trend with TGFβ1 treatment alone as well as with hypoxia alone (Figure 5.1B). Therefore, hypoxia has limited impact on the induction of genes associated with myofibroblast differentiation and ECM production.

At the protein level, TGFβ1 significantly increased α-SMA levels at 48- and 72-hours, with a noticeable induction at 24 hours (Figure 5.2A). A similar trend occurred with the induction of collagen 1, with the increase in response to TGFβ1 achieving significance at 72 hours compared

to all other treatments (Figure 5.2B). The protein level of FN 1 did not change significantly (Figure 5.2C). Hypoxia significantly reduced the expression of α-SMA (Figure 5.2A) and collagen I (Figure 5.2B) when cells were also exposed to TGFβ1. Thus, while we observed an increase at the mRNA level, markers of differentiation and ECM production in response to TGFβ1, hypoxia reduced the amount of protein production when increased by TGFβ1. Overall, these results show while TGFβ1 is crucial in the differentiation of fibroblasts into myofibroblasts, and that hypoxia may play an antagonistic role on the activating potential of TGFβ1.

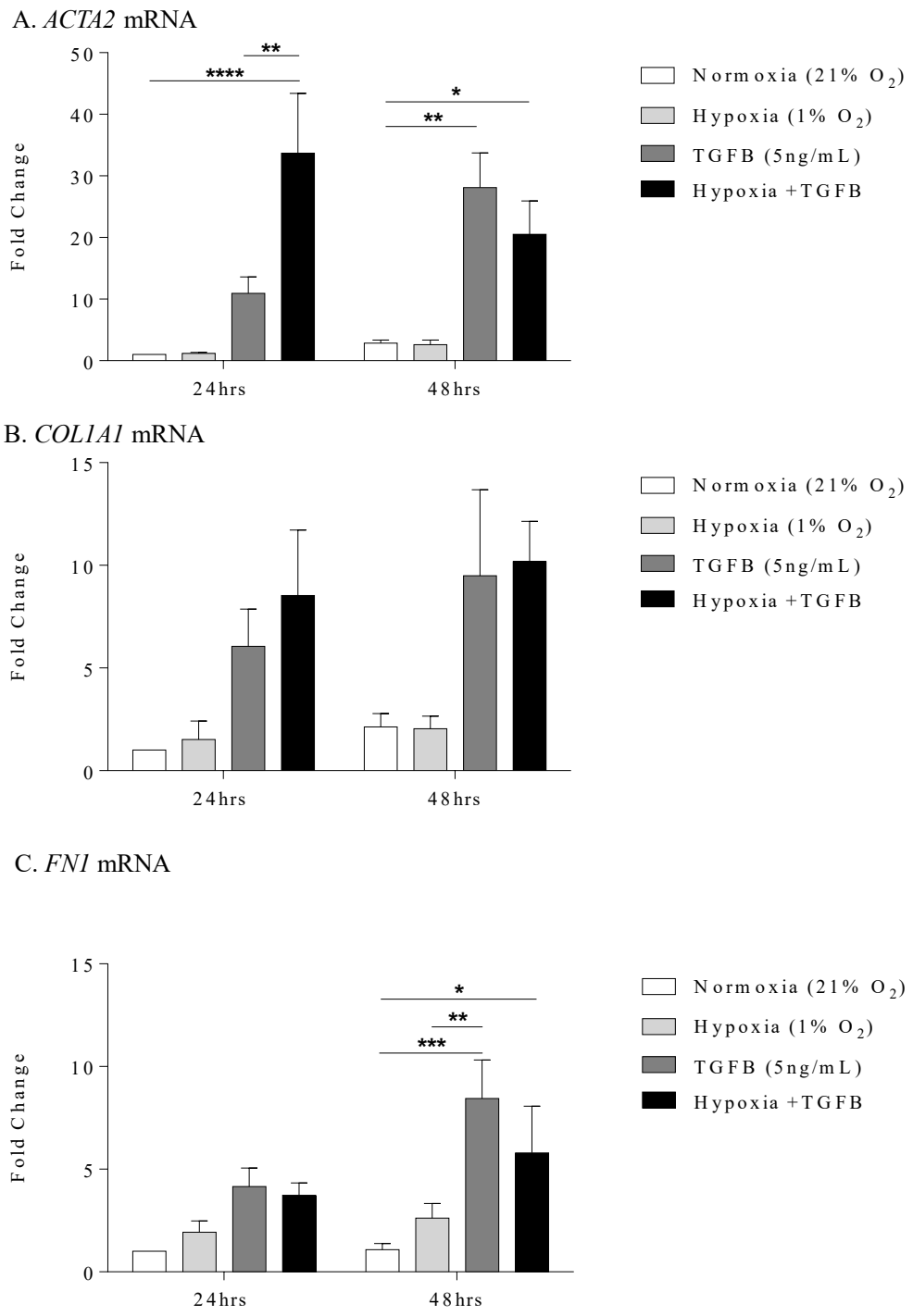


Figure 5.1: There is an increase in the mRNA of ACTA2, COL1A1, and FN1 in response to TGFβ1 and hypoxia with TGFβ1. (A) Levels of ACTA2 mRNA are signficantly increased by TGFβ1 (5ng/ml) at 48 hours and by hypoxia with TGFβ1 at 24- and 48-hours. The effect of TGFβ1 combined with hypoxia is significantly greater than TGFβ1 alone at 24- but not 48-hours. (B) The expression of COL1A1 mRNA do not significantly change, although an increasing trend was observed in response to TGFβ1 alone and when combined with hypoxia. (C) TGFβ1 increased FN1 mRNA levels significantly at 48 hours compared to normoxia and hypoxia, whereas TGFβ1

and hypoxia only increased FN1 mRNA levels significantly compared to normoxia at 48 hours. Values are represented as the mean ± SEM (n=4 independent experiments utilizing cells from 3 separate subjects). Means are expressed as fold change from the control (24h normoxia). *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$ compared to control (normoxia) unless otherwise specified.

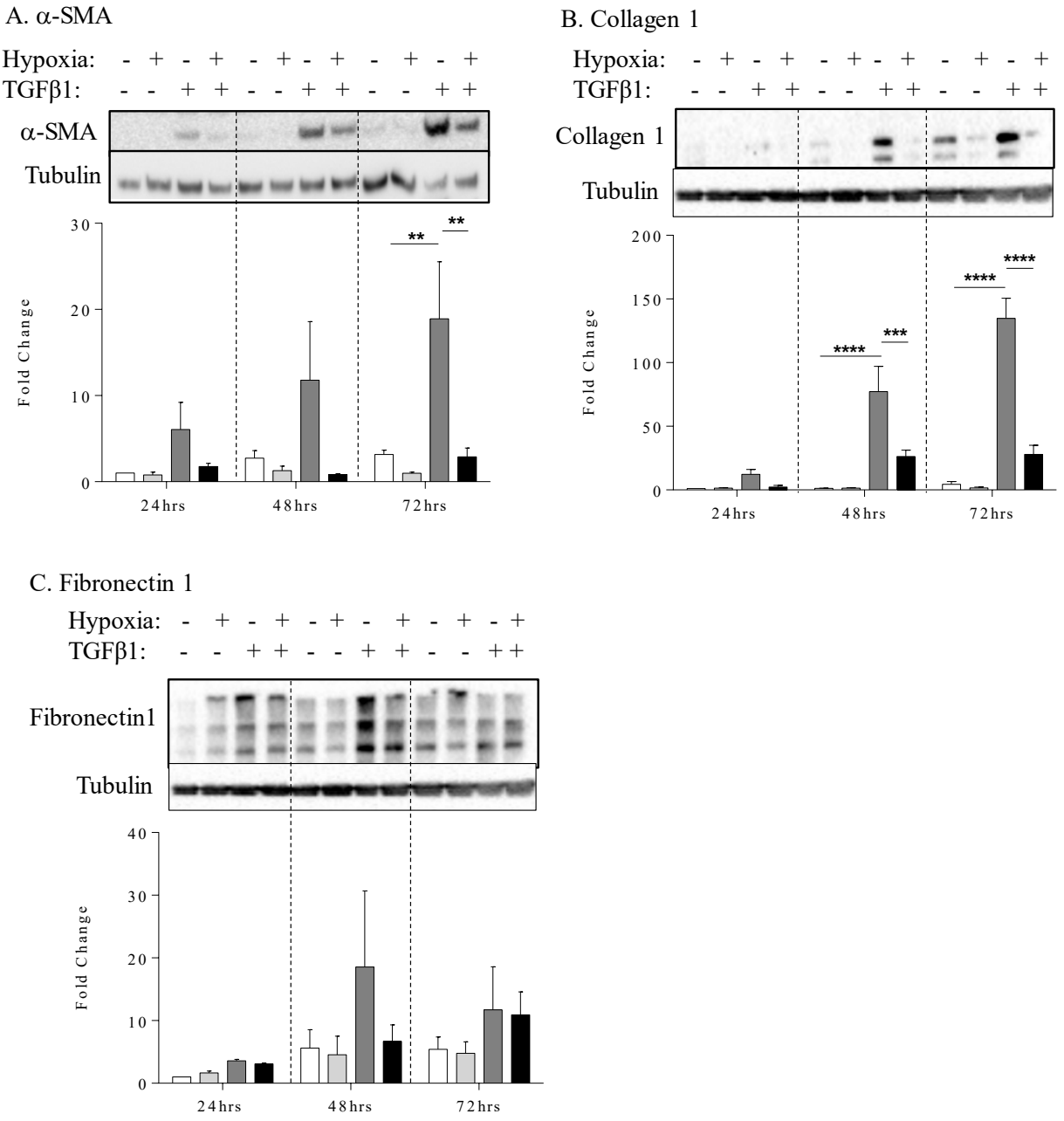


Figure 5.2: The TGFβ1-induced increase α-SMA and collagen 1 protein is attenuated by co-exposure to hypoxia. Upregulation of α-SMA (A) as well as collagen 1 (B) in HLFs occurred in a time dependant manner in response to TGFβ1 (5ng/ml). This increase is attenuated following inclusion of hypoxia (1% O_2). There is no significant change in fibronectin (C) protein levels in response to hypoxia, TGFβ1 or hypoxia with TGFβ1. Values are represented as the mean ± SEM (n=3-4 independent experiments, 3 separate subjects). Means are expressed as fold change from the control (24h normoxia). **p<0.01 and ****p<0.0001, as compared to control (48-, 72-h normoxia) unless otherwise specified.

5.2 Hypoxia attenuates TGFβ1-induced metabolic shift towards glycolysis in human lung fibroblasts.

As emerging evidence that metabolic reprogramming in IPF potentially drives disease pathogenesis, including current literature describing an increase in glycolysis and lactate in human lung tissue from IPF patients, we next focused on the effects of hypoxia with TGFβ1 on glucose metabolism in HLFs.[55] To investigate changes in metabolism, we first analyzed the mRNA expression of *LDHA* and *HK2*, the former being crucial in the conversion of pyruvate to lactate and the latter being one of three important regulatory enzymes in the glycolytic pathway. We then utilized ^{1}H NMR to analyze lactate production relative to glucose consumption. The combination of these analysis will allow us to gain a better understanding of glycolytic changes in HLFs in response to fibrosis-promoting conditions. At the mRNA level, there was a significant increase in *LDHA* mRNA only after treatment with hypoxia in conjunction with TGFβ1 at 48 hours, although there was an increasing trend in response to hypoxia and TGFβ1 alone (Figure 5.3A). We observed a similar, elevated tendency in *HK2* under conditions of hypoxia, and saw a significant increase at 48 hours under treatment with hypoxia and TGFβ1 (Figure 5.3B).

Next, we used ^{1}H NMR spectroscopy, and were able to measure two separate peaks, scaled in parts per million (ppm): glucose (5.2 ppm) and lactate (1.3 ppm). In order to quantify the rate of lactate secretion relative to the amount of glucose present in the cell-conditioned media, we used a known concentration of benzoic acid to establish a standard, followed by the integration of both the glucose and lactate peaks to calculate their individual concentrations. The relative integration of glucose and lactate compared to benzoic acid allowed us to determine the concentrations of each. The integrated concentration (mmol/l) is labeled in green above both peaks (Figure 5.4). When reading from left to right (Figure 5.4), the first peak denotes the concentration of glucose while the

following peak represents lactate concentration. We calculated the difference in glycolysis corresponding to each treatment at 24-, 48-, and 72-hours. We show ^{1}H NMR spectra (Figure 5.4A-4D) from the 72-hour time point only, as this period reflected the largest changes in lactate secretion. Our results reveal a significant increase in lactate production at 48- and 72-hours following treatment with TGFβ1 (Figure 5.4E). Moreover, we observed a significant decrease in lactate secretion when TGFβ1 is combined with hypoxia relative to TGFβ1 alone at 72 hours post-treatment. We do not observe any significant changes with hypoxia alone, although we observe a slight trend in increased lactate compared with normoxia. Thus, our results show that the shift in metabolism towards glycolysis caused by TGFβ1 that is attenuated under hypoxic conditions.

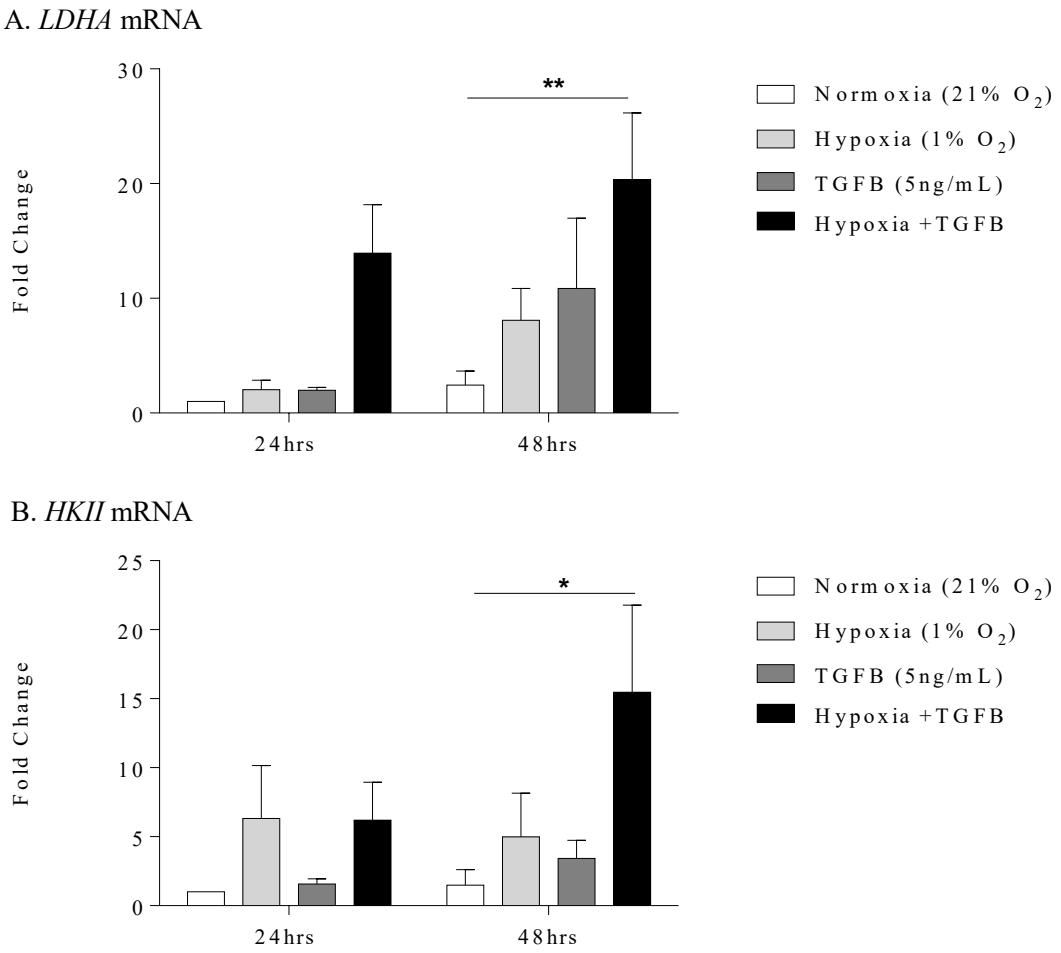
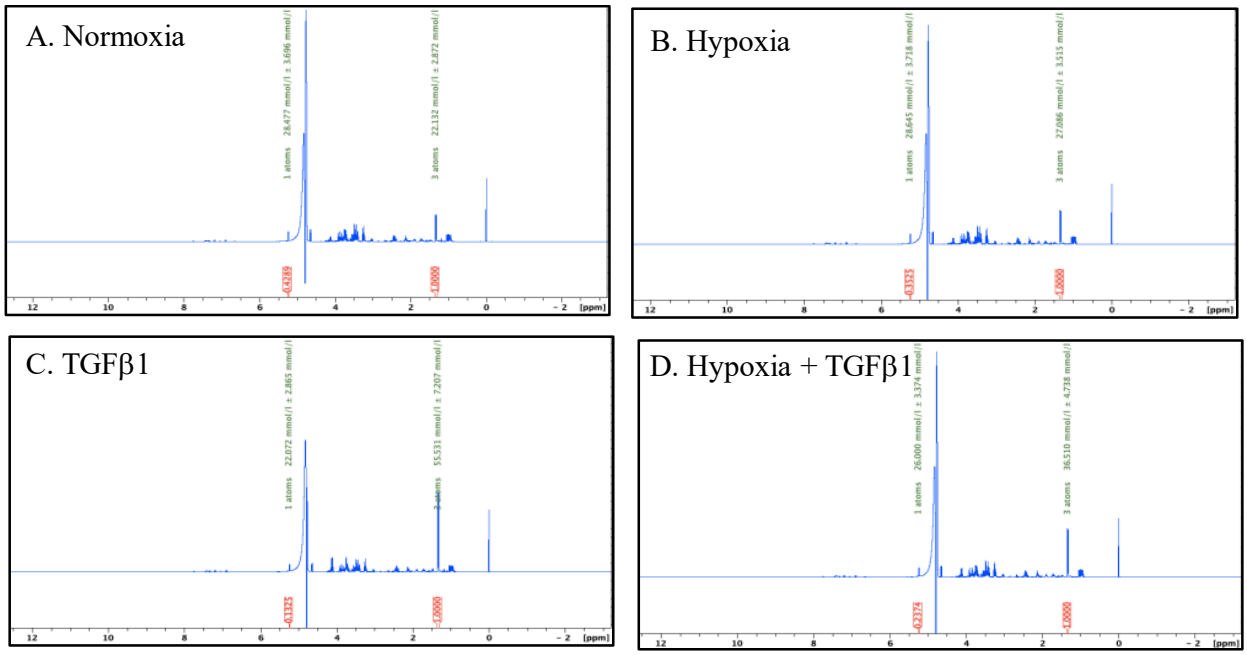


Figure 5.3: Increased mRNA levels of LDHA and HK2 following exposure to hypoxia with TGFβ1. There was a significant increase in LDHA (A) mRNA levels following treatment with hypoxia+TGFβ1 and a steady increase with hypoxia and TGFβ1 alone at 48 hours. Modest increase in HK2 (B) mRNA after treatment with hypoxia and steady increase with hypoxia+TGFβ1. Values are represented as the mean ± SEM (n=3-4 independent experiments, 3 separate subjects). Means are expressed as fold change from the control (24h normoxia). *$p<0.05$, **$p<0.01$, as compared to control (48h normoxia).



E. Lactate(1.3PPM)/Glucose (5.2PPM)

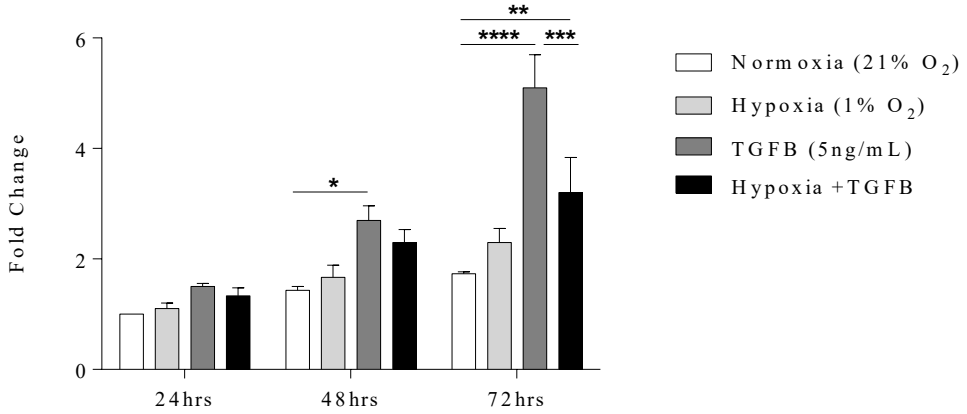


Figure 5.4: Hypoxia decreases lactate production induced by TGFβ1 at 72 hours. (A-D) Representative spectra from the 72-hour time-point for normoxia (A), hypoxia (B), TGFβ1 (C) and hypoxia with TGFβ1 (D). Lactate (1.3 PPM) and glucose (5.2 PPM) peaks were integrated in comparison to benzoic acid to determine the concentration (mmol/l), labelled in green (Figure A-D) of both molecules in every sample by ^{1}H NMR spectroscopy. (E) Quantification of the change in lactate concentration relative to glucose in a time dependant fashion. Values are represented as the mean ± SEM (n=3 independent experiments, 3 separate subjects). Means are expressed as fold change from the control (24h normoxia). *p<0.05, **p<0.01, and ****p<0.0001, as compared to control (48-, 72-h normoxia) unless otherwise specified.

5.3 Total mRNA and protein expression of HuR is does not change significantly following treatment with hypoxia, TGFβ1 or hypoxia with TGFβ1.

HuR plays a pathological role in many diseases, including liver and cardiac fibrosis.[69,93] However, a role for HuR in the context of IPF is not known. Previous literature has shown that HuR is elevated in mouse fibrotic liver sections at the mRNA and protein level.[95] Therefore, we first asked whether HuR expression changes in response to treatments that simulate conditions associated with IPF pathogenesis. As we observed attenuation by hypoxia at the level of differentiation and metabolic reprogramming, we were curious to see if hypoxia influenced HuR expression. However, neither hypoxia, TGFβ1 or hypoxia with TGFβ1 significantly changed the expression of HuR at both the mRNA (Figure 5.5A) or protein levels (Figure 5.5B). Thus, under conditions that cause fibroblast differentiation and ECM production, there is no change in HuR levels in lung fibroblasts.

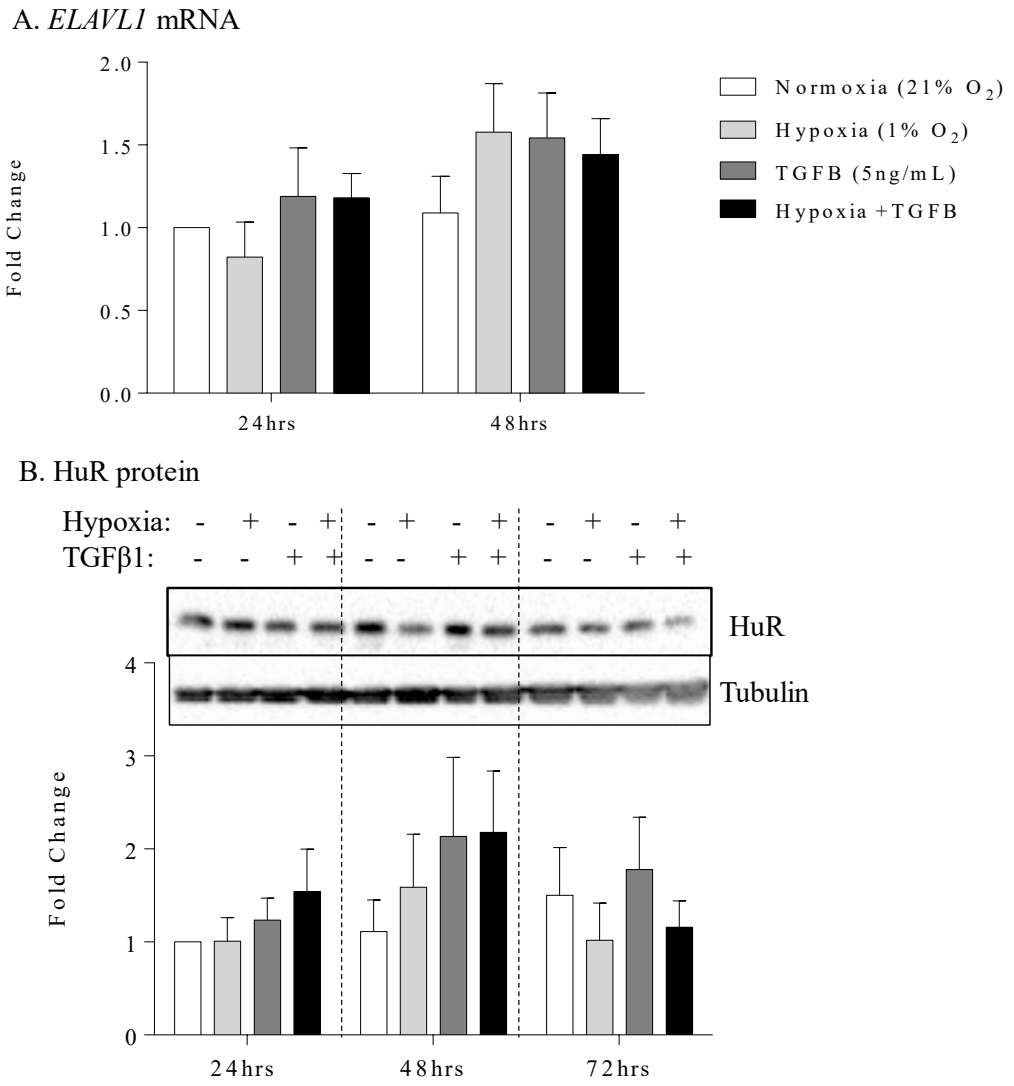
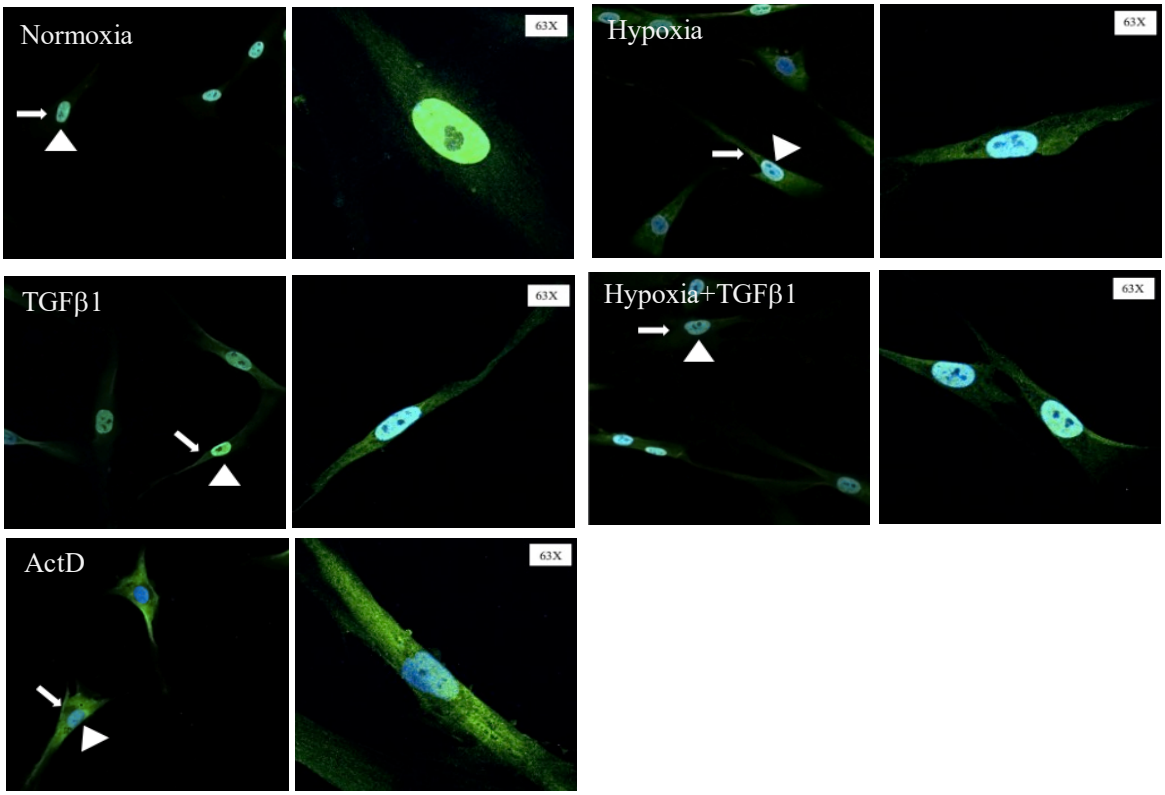


Figure 5.5: HuR expression remains unchanged in response to hypoxia, TGFβ1 and hypoxia with TGFβ1 in HLFs. (A) mRNA levels and (B) protein levels of HuR in HLFs were assessed 24-72 hours following treatment with hypoxia (1% O_2), TGFβ1 (5ng/ml) and hypoxia with TGFβ1. Values are represented as the mean ± SEM (n=4-6 independent experiments, 3 separate subjects). Means are expressed as fold change from the control (24h normoxia)

5.4 HuR translocation to the cytoplasm is increased following treatment with hypoxia, TGFβ1 and hypoxia together with TGFβ1.

A major function of the HuR lies in its ability to stabilize target mRNA in the nucleus and translocate to the cytoplasm, thereby ensuring mRNA stability by providing protection from degradation machinery, which leads to increased half-life of the mRNA or increased association with polysomes, ultimately leading to increased translation.[70,71] The effect of hypoxia, TGFβ1 or hypoxia with TGFβ1 on HuR localization is currently unknown in HLFs. Therefore, we treated primary normal HLFs from three separate subjects with hypoxia, TGFβ1 or hypoxia with TGFβ1 for 4 hours and utilized IF microscopy to assess the cellular localization of HuR. Figure 5.6A illustrates that under basal conditions, HuR is predominantly nuclear (*arrowheads*), with very little cytoplasmic localization (*arrows*). Moreover, we observe higher cytoplasmic levels of HuR under hypoxic treatment (Figure 5.6A). Similarly, cytoplasmic HuR is elevated in fibroblasts treated with TGFβ1 alone (Figure 5.6A). An increase in cytoplasmic HuR can also be seen in fibroblasts treated by hypoxia combined with TGFβ1 (Figure 5.6A). As a positive control, Actinomycin D (ActD) induced almost complete translocation of HuR from the nucleus to the cytoplasm (Figure 5.6A).[86] Furthermore, we were able to quantify cytoplasmic localization of HuR by comparing fluorescence of the cytoplasmic fraction of HLFs subtracted nuclear fraction. There was a significant increase in cytoplasm localization of HuR under hypoxia, TGFβ1 as well as hypoxia with TGFβ1 compared to normoxia (Figure 5.6B). There was less translocation of HuR to the cytoplasm in response to hypoxia with TGFβ1 compared to either hypoxia or TGFβ1 alone (Figure 5.6E). In summary, HuR translocates to the cytoplasm in response to hypoxia, TGFβ1 and hypoxia with TGFβ1; however, the level of translocation is greater under hypoxia or TGFβ1 alone compared to the combined treatment.

A. HuR Localization



B. HuR Quantification IF

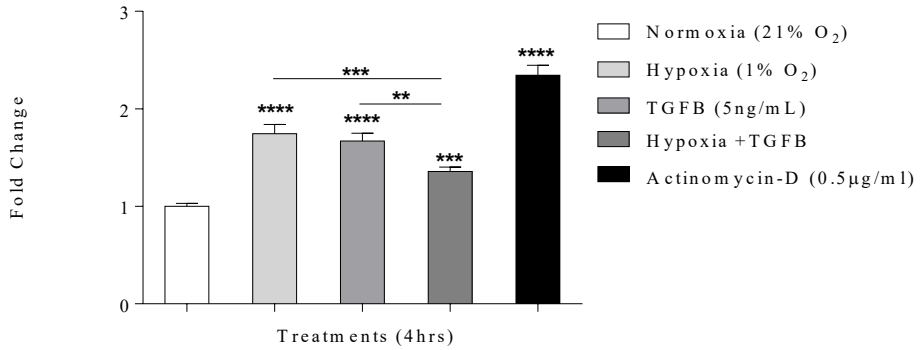


Figure 5.6: HuR translocation to the cytoplasm is increased in response to hypoxia, TGFβ1 and hypoxia with TGFβ1. (A) HuR localization using immunofluorescence at 20x magnification (left panels) shows cytoplasmic translocation in HLFs treated with hypoxia (1% O_2), TGFβ1 (5ng/ml) and hypoxia with TGFβ1. White arrowheads designate blue nuclear staining (Hoescht) while white arrows indicate green cytoplasmic staining. Note the 63x magnification images (right panels) showing representative cells and associated HuR localization. ActD was used as a positive control for HuR translocation to the cytoplasm. (B) HuR quantification of immunofluorescence. Values are represented as the mean ± SEM (n=3-4 independent experiments, 3 separate subjects). Means are expressed as fold change from the control (4h normoxia). ***p<0.001, and ****p<0.0001, as compared to control (4h normoxia).

5.5 HuR is required for fibroblast differentiation and the production of collagen 1 in response to TGFβ1.

HuR increases fibroblast differentiation as well as collagen synthesis in liver fibrosis.[69] Therefore, our next question revolved around the involvement of HuR in fibroblast differentiation and ECM production in the lung. To test this, we reduced HuR levels in using siRNA targeting HuR, which resulted in a decrease of more than 75% in total HuR protein levels (Figure 5.7A). This reduction in HuR significantly attenuated the TGFβ1-induced increase in α-SMA and collagen 1 protein levels (Figure 5.7B and 5.7C). HuR knockdown also significantly decreased both α-SMA and collagen 1 protein levels in HLFs treated with hypoxia and TGFβ1. While we see a decreasing trend in FN1 upon knockdown of HuR under TGFβ1 alone and combined with hypoxia, this did not reach statistical significance (Figure 5.7D). These data indicate that a HuR plays an important role in promoting fibroblast differentiation and collagen 1 production.

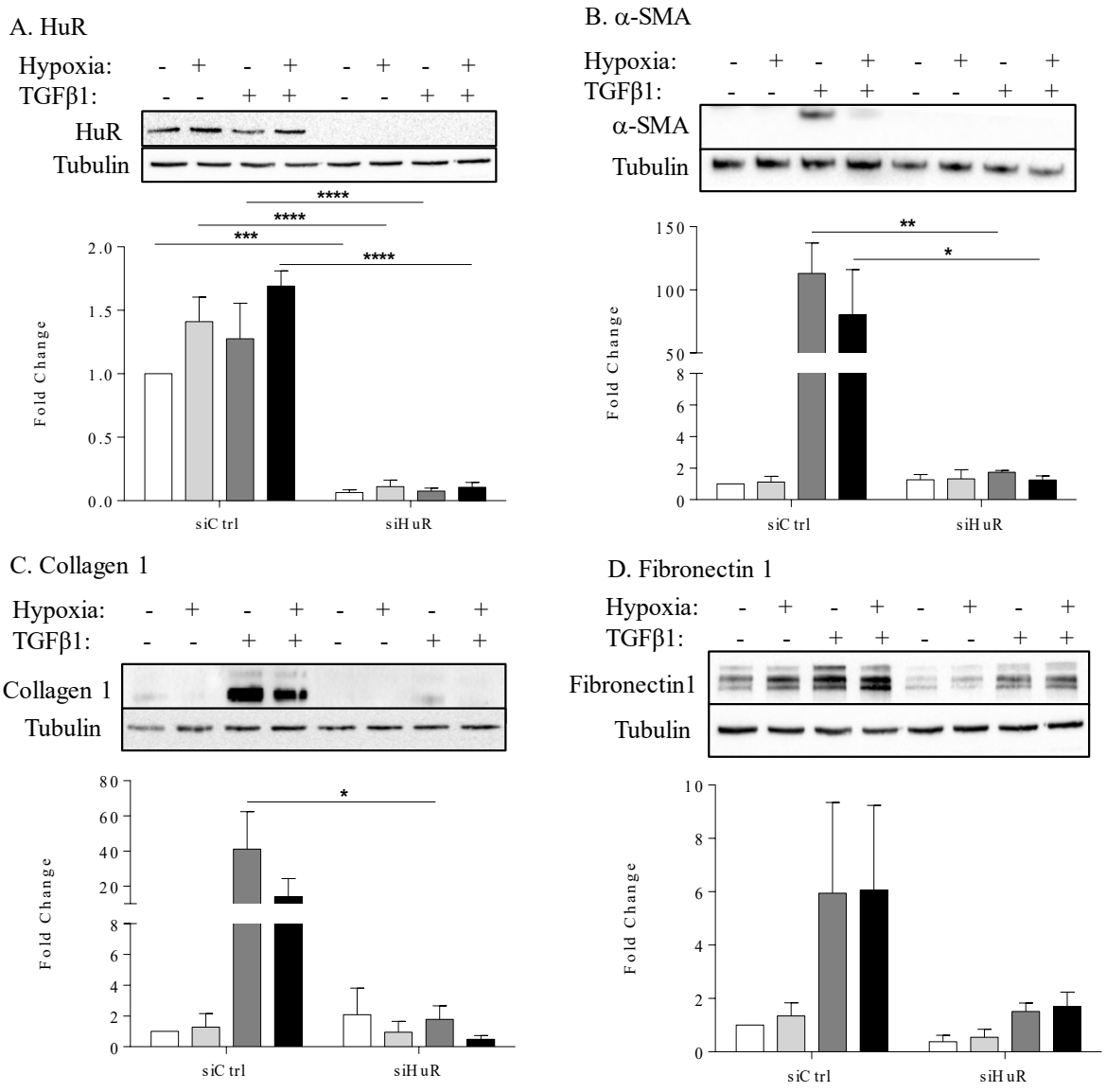
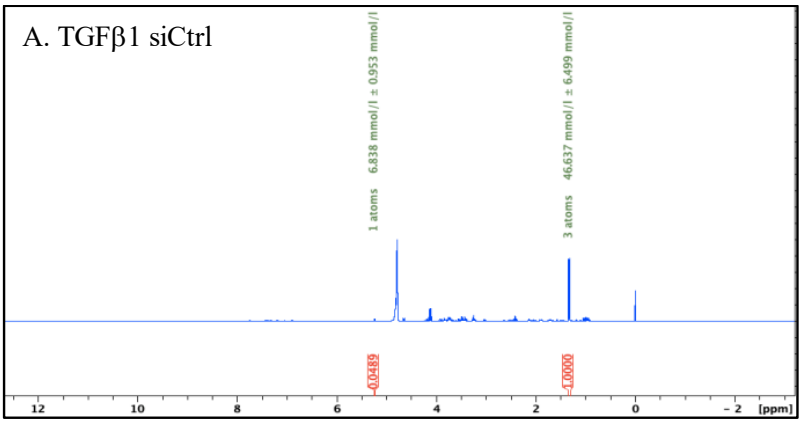
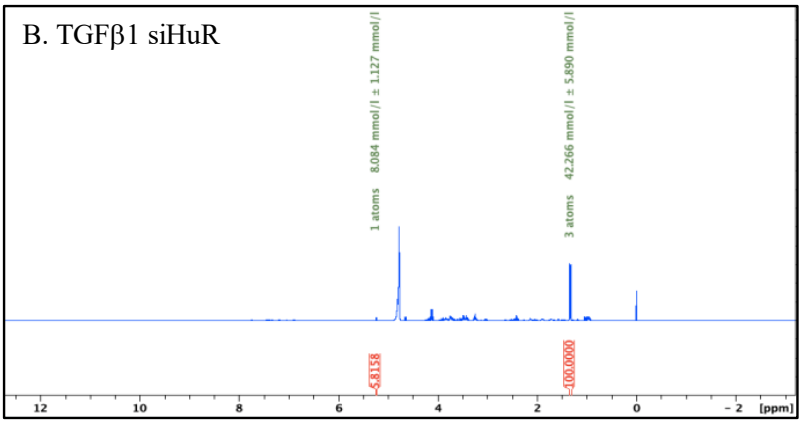


Figure 5.7: HuR knock-down attenuates TGFβ1-induced increase of α-SMA and collagen-I in HLFs. (A) HuR protein levels in HLFs transfected with siRNA targeting HuR (siHuR) and siRNA scrambled control (SiCtrl). TGFβ1-induced increase in protein levels of α-SMA (B) and collagen I (C) were downregulated significantly following HuR knock-down. (D) Fibronectin I showed no significant changes at the protein level following HuR siRNA transfection. Values are represented as the mean ± SEM (n=3-4 independent experiments, 3 separate subjects). Means are expressed as fold change from the control (siCtrl normoxia). *p<0.05 and **p<0.01 as compared to control siRNA for each treatment.

5.6 HuR does not play a significant role in a TGFβ1-induced metabolic shift

Because our data indicate that HuR plays a role in TGFβ1-induced differentiation and ECM production, we next investigated the role of HuR in the metabolic shift. After transfecting HLFs with HuR siRNA or control siRNA and treating with hypoxia, TGFβ1 and hypoxia with TGFβ1 for 72 hours, we utilized ^{1}H NMR to measure lactate production versus glucose consumption. Figure 5.8A and 5.8B represent sample spectra from TGFβ1-treated HLFs and demonstrate minimal changes in lactate production after HuR knock-down. At the level of lactate production in response to hypoxia and hypoxia with TGFβ1, we did not observe significant changes compared with normoxia (Data not shown). Figure 5.8C represents quantification of lactate production, showing that HuR does not play a significant role in increasing glycolytic flux in HLFs. Thus, while HuR reduces TGFβ1 induction of fibroblast differentiation and ECM production, there is no significant change at the level of TGFβ1-induced glycolysis. Overall, HuR does not play a significant role in metabolic reprogramming in HLFs





C. Lactate (1.3PPM)/Glucose (5.2PPM)

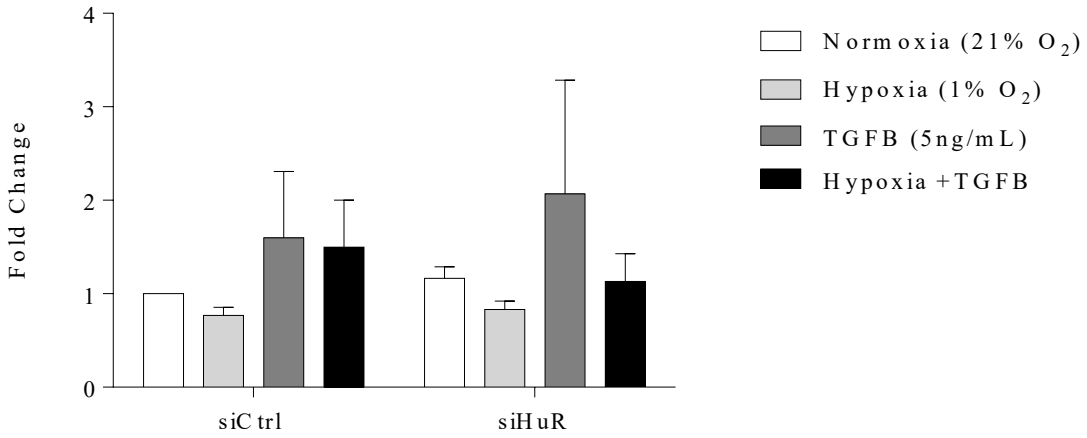


Figure 5.8: Lactate production does not change significantly in response to HuR knock-down in HLFs. (A-B) Sample spectra from HLFs transfected with siCtrl and siHuR that were treated with TGFβ1. Note the similar lactate and glucose peaks at 5.2ppm and 1.3ppm respectively. (C) Quantification of Lactate concentration relative to glucose between siCtrl mRNA and siHuR mRNA transfected HLFs. Values are represented as the mean ± SEM (n=3 independent experiments, 3 separate subjects). Means are expressed as fold change from the control (siCtrl normoxia). Values are compared to the control siRNA for each treatment.

Chapter 6 DISCUSSION

IPF is a devastating and progressive disease that typically leads to fatal respiratory failure, with a median survival of 3-5 years from diagnosis[2]. The exact cause of IPF remains unknown and the underlying mechanisms behind disease pathogenesis are poorly understood.[36] One of the main features of IPF is TGFβ1-driven fibroblast-to-myofibroblast differentiation, which is characterized by the expression of a-SMA and increased collagen I production.[3] In addition, an emerging feature of IPF is metabolic reprograming in lung tissue that leads to an increase in aerobic glycolysis and lactate production, resulting in pH-dependent activation of TGFβ1.[52,55] The presence of HIF-1α further contributes to myofibroblast differentiation caused by metabolic reprogramming,[96] but the mechanistic factors that govern these phenomena, ultimately leading to fibroblast differentiation, are not well understood. In the following study, we focused on a novel link between myofibroblast differentiation, metabolic reprogramming and the RNA-binding protein HuR. Current literature shows that HuR regulates the stability and/or translation of target mRNA that encode proteins involved in pathogenic mechanisms that drive disease, of which cell differentiation is one. In support of this, experimental studies show that silencing HuR attenuates differentiation by reducing collagen and α-SMA expression in hepatic stellate cells (HSCs), as HSCs are the primary source of myofibroblasts in liver cirrhosis.[69,95] In the context of a metabolic shift, previous studies have suggested that HuR upregulates *HIF1A* mRNA and increases its translation.[70,97] Moreover, HIF-1α, which is well-known to promote glycolysis, is elevated in IPF lung samples.[64] Therefore, we sought to evaluate whether HuR contributes to the pathogenesis of IPF by regulating mRNA and proteins of pro-fibrotic and glycolytic pathway markers in primary lung fibroblasts by simulating IPF microenvironment conditions through treatment with TGFβ1 and hypoxia (Figure 6.1). Herein, we have described that HuR is important in controlling fibroblast differentiation and consequent ECM production.

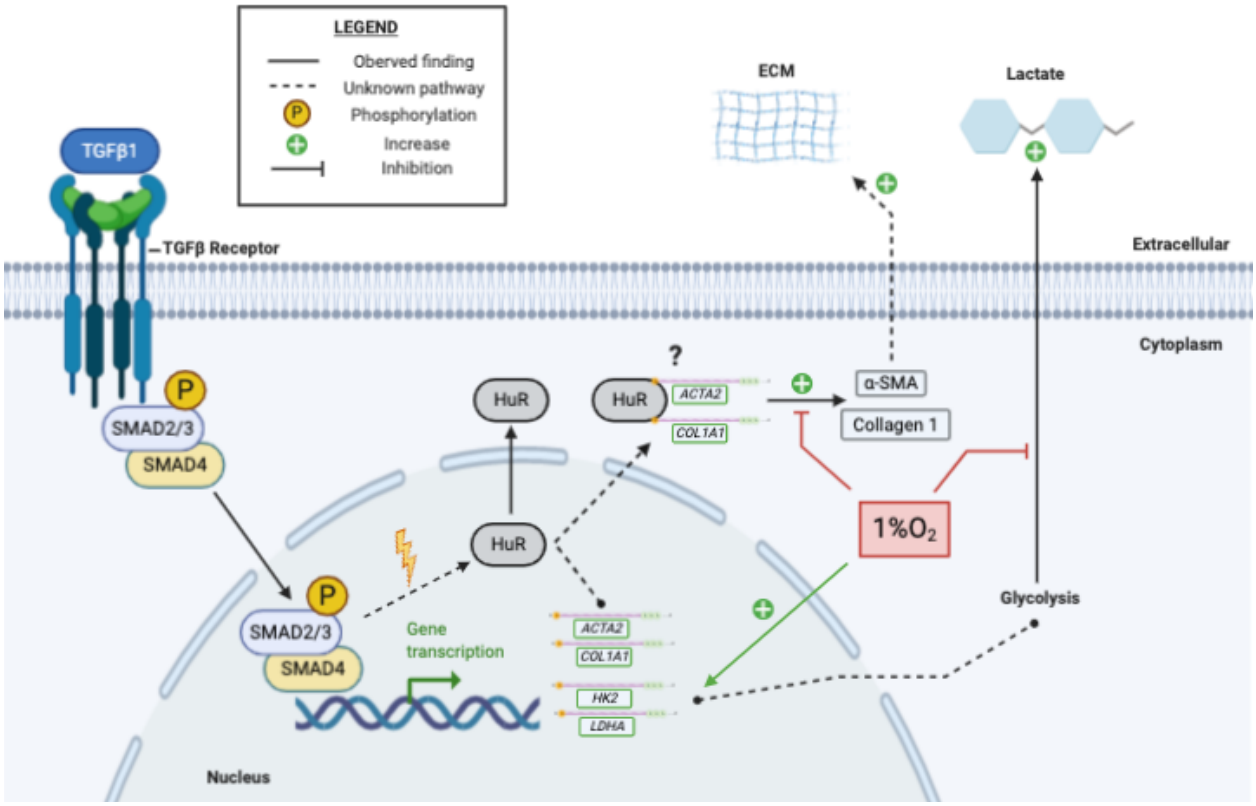


Figure 6.1: A schematic representation of the role of HuR in human lung fibroblasts in response to TGFβ1 and hypoxia exposure. In response to TGFβ1 and hypoxia, we observed increased HuR translocation to nucleus. HuR is crucial in the process of fibroblast differentiation and collagen production, however, the pathway by which it interacts with α-SMA and collagen 1 is unknown. Furthermore, the role of hypoxia is represented as it greatly reduced TGFβ1-induced fibroblast differentiation and lactate production, despite increasing HK2 and LDHA. Information adapted from Mingyuan et al. [61] and Sgalla et al. [3].

One of the more intriguing findings from this work was the combined effect of hypoxia and TGFβ1 on markers of myofibroblast differentiation and metabolic reprogramming, as these two key pillars are involved in the pathogenesis of IPF[52,58,96]. In this study, we hypothesized that HuR would drive both the differentiation and metabolic reprogramming in response to hypoxia and TGFβ1 by stabilizing mRNAs of ECM and as well as those recognized as glycolysis markers. Our rationale was the fact that HIF-1α is upregulated in IPF; that hypoxia is a well-known inducer of HIF-1α; and the presence of hypoxia or hypoxia-induced signalling through the HIF-1α pathway underlies the pathogenesis of various life threating lung diseases.[60,98] For instance, hypoxia

resulting from organ ischemia leads to increased expression of pro-inflammatory cytokines and chemokines.[99] Chronic hypoxia in the lung has been linked with several pathological processes such as vascular proliferation, increased vascular reactivity, chronic pulmonary hypertension and right heart failure.[100] In lung cancer, hypoxia is closely related to fundamental changes in tumor cells, resulting in resistance of cell death, increased angiogenesis and reprogramming energy metabolism. In cancer cells found within a tumor, HIF-1α induces the overexpression of GLUT1 and the metabolic enzymes HKII and LDH, the net result of which increases glucose uptake and the switch to aerobic glycolysis.[101]

While the role of hypoxia and hypoxia-induced HIF-1α in IPF are unknown, two important studies highlight a potential the link between HIF-1α and IPF. These studies have revealed that over-expression of HIF-1α enhances TGFβ1 activity, thereby increasing myofibroblast differentiation.[52,96] Although these studies suggest an important pathological role for hypoxia and HIF-1α in IPF, the mechanism driving this increased differentiation remains poorly understood. With this rationale, the first step was to understand whether hypoxia affected differentiation and metabolic regulation in HLFs. We observed that hypoxia- when combined with TGFβ1- increased *ACTA2* mRNA compared to TGFβ1 alone. This effect was time-dependent, as the induction of *ACTA2* mRNA was seen only at 24 hours; by 48 hours post-treatment the effect of hypoxia in conjunction with TGFβ1 was not different from hypoxia alone. We do not believe the decrease after 24 hours to be a result of increased cell death, as there was no difference in cell viability between normoxia and hypoxia-treated cells (data not shown). We speculate that this response may reflect a self-defence mechanism which fibroblasts use to control excess expression of *ACTA2* mRNA. The effect of hypoxia together with TGFβ1 on *ACTA2* mRNA was mirrored at the protein level, whereby there was also significantly less α-SMA expression. Therefore, hypoxia exposure

alters fibroblast differentiation and consequent ECM production. Overall, within the initial 24 hours of exposure, hypoxia appears to quickly exacerbate the process of differentiation in fibroblasts as seen by the transcriptional levels of *ACTA2*. However, this is followed by an unknown mechanism that slows the transcription of *ACTA2* which ultimately results in the lower levels of α-SMA protein. Moreover, the apparent decrease in differentiation- based on α-SMA- is also seen at the protein level for collagen I, which is significantly decreased in a similar level to α-SMA by hypoxia.

Concurrent with this, we also evaluated whether hypoxia would regulate mRNA and proteins that are important in metabolic reprograming. Upon exposure to only 1% O_2 (thereby mimicking hypoxic conditions), there was a trend of induction of *LDHA* mRNA, *HK2* mRNA and lactate production. However, this induction did not reach statistical significance in response to hypoxia alone. In contrast, hypoxia combined with TGFβ1 did result in a statistically significant increase in *HK2* mRNA and *LDHA* mRNA. This was surprising, as we had hypothesized that extracellular lactate production would increase in response to hypoxia alone, given the established association between hypoxia and increased production of lactate as a result of HIF-1α activation.[102] Moreover, previous work has shown that HIF-1α, when induced by hypoxia, leads to increased LDH induction and myofibroblast differentiation.[52] It is possible that 1% O_2 is not sufficient to induce lactate production- on its own- in primary normal HLFs used in our study. Furthermore, previous literature demonstrates fibroblasts uptake greater levels of lactate from media compared with mesenchymal stem cells.[103] Therefore, increased lactate uptake may overcome the level of hypoxia cells are exposed to. However, we did observe a significant increase in lactate in response to TGFβ1, an effect that could be mediated by HIF-1α. A recent study showed that a knockdown HIF-1α abrogates TGFβ1-induced lactate production in primary

fibroblast derived from IPF subjects.[96] Interestingly, and similar to the ECM markers, our data show that exposure to hypoxia reduces TGFβ1-induced lactate production at 72 hours. Based on this result, we would anticipate that the protein level of the enzyme LDHA, responsible for converting pyruvate to lactate, would also be reduced, thereby explaining the decrease in lactate production despite the increase in *LDHA* mRNA. Furthermore, it appears that hypoxia slows down the general effect caused by TGFβ1 (*e.g.* differentiation and metabolic reprogramming), presumably to a minimal level, to allow for longer periods of survival. This is supported by evidence that hypoxia decreases phosphorylation of SMAD-2 and SMAD-3 proteins, in macrophages and cardiac myocytes, which is important in the TGFβ1 signalling pathway.[104,105] Thus, we consider the possibility that a decrease in phosphorylation potential could be affecting components of the TGFβ1 pathway, such as SMAD proteins, which are known to be tightly controlled by phosphorylation and dephosphorylation events.[106] In summary, we propose that hypoxia reduces the ability of TGFβ1 to induce a maximal response, whether it be the process of differentiation or reprogramming, as a result of an overall energy decrease.

The mechanism through which hypoxia reduces fibroblast differentiation, ECM levels and lactate production induced in response to TGFβ1 is not known, but there are several possibilities. First, this may involve the RhoA GTPase signalling network. RhoA regulates the serum response factor (SRF) and myocardin related transcription factor (MRTF) signalling pathway, which is crucial for fibroblast-to-myofibroblast differentiation.[107,108] Recent literature has demonstrated that TGFβ1 stimulates RhoA, allowing it to activate MRTF/SRF complex. Once the MRTF/SRF complex is activated, it induces the transcription of target genes, including α-SMA and collagen.[109-111]. Interestingly, RhoA is inhibited by ARHGAP29, which is increased following hypoxia exposure.[112,113] ARHGAP29 belongs to a class of regulatory proteins which leads to RhoA

inhibition.[113] Furthermore, hypoxia induces the transcriptional up-regulation of adenyl cyclases that inhibit RhoA activity in a variety of carcinoma cell lines.[114] Altogether, these findings show evidence that TGFβ1-induction of α-SMA and collagen I is reduced by hypoxia via a reduction in RhoA activity.

Role of HuR

Another possibility- and the focus of this body of work- in understanding whether hypoxia controls the differentiation of lung fibroblasts via HuR. In general, HuR is associated with stabilizing several target mRNA, many of which encode proteins involved in cell growth, cancer and inflammation.[67] Experimental data has shown that HuR stabilizes mRNA primarily containing AREs.[71] Researchers have found that HuR protects mRNA from degradation machinery, rather than slowing the rate of deadenylation, in experiments where HuR is overexpressed.[115] In addition, overexpression of HuR may act to stabilize its target mRNA by actively sequestering other factors needed for degradation, thereby preventing recruitment to the exosome.[71] HuR has also been implicated in promoting the translation of various target mRNA such as HIF, VEGF and cyclin A2.[67] It should be noted that HuR also has the ability to decrease mRNA stability and/or translation in response to various stimuli.[116-118] An example of mRNA destabilization by HuR is seen during the early steps of myogenesis, whereby HuR reduces the expression of the cell cycle promoter nucleophosmin (NPM).[118] Moreover, HuR collaborates with another RNA-binding protein known as the KH-type splicing regulatory protein (KSRP) to form a complex to maintain low levels of *NPM1* mRNA. This association with KSRP demonstrates that HuR can collaborate with other RNA-binding proteins in order to destabilize mRNA and thus reduce protein expression.[118] HuR can also regulate levels of the telomere-associated protein (TIN2) by both destabilizing *TIN2* mRNA and reducing its translation. Conversely, depletion of HuR enhanced TIN2 expression.[116]

Thus, while HuR is typically associated with mRNA stability and increased translation of its targets, these studies provide evidence that HuR can also negatively regulate mRNA and protein expression, depending on the stimuli and the association with protein partners. In our study, we initially proposed that hypoxia would alter HuR activity and lead to a decrease in markers of differentiation and metabolic reprogramming. Previous literature has shown that during hypoxia, global transcriptional activity is reduced as a result of post-transcriptional modifications that target mRNA turnover and translational control.[119] These processes are efficiently controlled by HuR.[119] During hypoxia, there is evidence of post-translational modifications of HuR, including phosphorylation by kinases checkpoint kinase 2 (Chk2) and protein kinase C (PKC). Chk2 and PKC are activated during hypoxia.[78,120] In addition, Chk2 modulates HuR function by phosphorylating the RNA-recognition motifs RRM1 and RRM2 on HuR, thereby changing its interaction with mRNA targets.[121] Ultimately, hypoxia, as a source of stress, has been implicated in modifying the function and behaviour of HuR. We propose that hypoxia may alter HuR function in HLFs, which may be attributed to post-translational modifications, leading to a reduction of ECM protein levels by reducing either the mRNA stability and/or their translation.

To first determine whether HuR could control- or even drive- cell differentiation and metabolic reprogramming, we evaluated its expression and localization in response to TGFβ1 and hypoxia. Exposure of HLFs to the hypoxia, TGFβ1 or both did not change significantly the total expression of HuR. However, it is established that HuR translocation to the cytoplasm, rather than change in its expression, lead to functionals changes in the cell.[67] This is supported by a report for example demonstrating that exposure to UV light did not significantly induce the total HuR expression, but induced its translocation to the cytoplasm, where HuR then stabilized *p21* mRNA.[93] Because considerably less is known about the effect of hypoxia and TGF-β on the localization of HuR in HLFs,

we evaluated the ability of these exposures to cause shuttling of HuR from the nucleus where it normally resides to the cytoplasm. Our data show the localization of HuR in the cytoplasm is increased in response to both hypoxia and TGFβ1 alone. This is in agreement with a previous study whereby TGFβ1 increased HuR shuttling to the cytoplasm in cardiac fibroblasts.[93] Hypoxia exposure has been also shown to increase cytoplasmic HuR in a time-dependant manner in cancer cells.[122] Although the mechanism behind TGFβ1 and hypoxia-induced cytoplasmic HuR translocation is unknown, we propose that it may involve the activation of the p38 mitogen-activated protein kinase (MAPK) pathway. TGFβ1 activates p38 MAPK, and inhibition of this pathway prevents translocation of HuR to the cytoplasm.[69,93] Hypoxia can also induce p38 MAPK activity and phosphorylation of HuR by p38 MAPK alters its subcellular localization and/or its binding to target mRNA.[67,123] Interestingly, p38 transduces cytokine and mechanical signals into myofibroblast differentiation through SRF and inhibition of myofibroblasts was observed in mice with a deletion of Mapk14.[124] This raises the possibility that TGFβ1 and hypoxia-induced HuR translocation may be mediated through the p38 MAPK signalling pathway.

HuR translocation to the cytoplasm suggests that it could be controlling the expression of ECM markers and lactate secretion induced by TGFβ1; this hypothesis was verified by silencing HuR. Indeed, knock-down HuR in HLFs significantly attenuates TGFβ1 induction of α-SMA and collagen I protein. Interestingly, our results showed that following HuR knock-down, HLFs treated with siHuR demonstrated similar total protein levels of α-SMA and collagen 1 across all treatment conditions. Thus, we consider the absence of HuR to be of greater importance in reducing the process of fibroblast differentiation in comparison to the decrease seen with hypoxia. One may consider HuR a master regulator that is crucial for driving the process of myofibroblast differentiation forward. Moreover, HuR deficiency is linked with a loss of stress fibres due to

impairment of the actin cytoskeleton.[125] This could be crucial, as the rearrangement of the actin cytoskeleton is known to liberate MRTF from G-actin and co-associate with SRF in order to induce gene transcription leading to differentiation.[109] Increased RhoA activity is associated with enhanced stress fibre formation, therefore loss of these stress fibres due to HuR depletion could be involved in RhoA inactivation and ultimately decreased differentiation in HLFs.[112]

It is of interest that silencing HuR did not alter levels of secreted lactate in HLFs as it did for myofibroblast markers. These results suggest that the ability of TGFβ1 to cause lactate secretion is not controlled by HuR. It is possible that TGFβ1 may be activating on enzymes in the glycolytic pathway directly, such as HKII and LDHA, in order to increase in lactate secretion.[126] Thus our data suggest that HuR plays a negligible role in regulating mRNA and/or enzymes involved in the glycolytic pathway. This is based on our evidence that TGFβ1 increased lactate production and that there was no significant change in lactate secretion between lung fibroblasts treated with siHuR and siCtrl. However, our results strongly suggest that HuR is playing a crucial role in the differentiation of fibroblasts to myofibroblasts. Future experimentation addressing HuR stability of target mRNA involved in differentiation could shed light on the direct involvement of HuR at the RNA level. One might predict that HuR is playing a role in the translation of α-SMA and collagen 1, a prediction could be addressed via evaluating polysome fractions in mRNA-protein complexes to determine rates of protein biosynthesis.[70] It is also feasible that under certain conditions, such as hypoxia, HuR is sequestering mRNA in stress granules (SGs).[117,127,128] SGs are ribonucleoprotein complexes that contain stalled mRNAs, pre-initiation factors and specific RNA binding proteins which allow cells to adapt and respond to stress.[128,129] Recent studies have shown that cellular hypoxia leads to the phosphorylation of eIF2α, ultimately resulting in the accumulation of the RNA pre-initiation complex 48S and promoting the formation of SGs.[130,131]

SGs contain HuR in addition to other RBPs that selectively bind to target mRNA with high ARE content. In a study performed on human osteoarthritis (OA) chondrocytes, *Cox-2* mRNA was sequestered in SGs by HuR in response to IL-1β.[117] Formation of SGs in chondrocytes resulted in the protein level of COX-2 decreasing because of delayed translation of *Cox-2* mRNA. In contrast, when chondrocytes were treated with siHuR, *Cox-2* mRNA did not translate to SGs and protein levels were increased early in response to IL-1β.[117] It has been suggested that SGs may play a role in keeping mRNA in translationally-silent state. Formation of SGs has been associated with sequestering key components of well-established pathways such as the NF-κB and p38/JNK pathways in response to hypoixa.[132] Thus, in response to hypoxic stress on HLFs, HuR may be involved in sequestering *ACTA2* and *Col1a1* mRNA in SGs, thereby reducing their translation and subsequent expression.

Limitations and Future Directions

Our work is the first to elucidate the relationship between HuR and fibroblast differentiation in primary HLFs using a model that exposes cells to TGFβ1 and hypoxia, a combined condition that is thought to replicate conditions seen in the more complex microenvironment of the lungs. One of the limitations of our study was that we only explored the effects of TGFβ1 and hypoxia *in vitro* in normal HLFs. Furthermore, experiments in this study consisted of 3-6 replicates from 3 separate human subjects, therefore conclusions should be seen as tentative. It would be of interest to confirm our results in HLFs harvested from human IPF lung tissue. Despite this, our study strongly supports the notion that HuR cytoplasmic translocation is elevated in response to TGFβ1 and hypoxia in HLFs. We also showed that HuR regulates TGFβ1-induced lung fibroblast differentiation. Finally, we found that hypoxia can abrogate TGFβ1-induced differentiation in HLFs. In this context, it would be interesting to study the effects of

TGFβ1 and hypoxia in an *in vitro* model such as a scaffold-free epithelial-fibroblast coculture in order to further replicate the microenvironment for HLFs.[133] Epithelial cells lining the alveolar membrane are thought to be the starting point for fibrosis development as they have the ability to secrete several pro-fibrotic factors in addition to TGFβ1.[3] Furthermore, an important future experiment would be to study the role of HuR and IPF *in vivo*. It should be noted that HuR is well-established in organ development, therefore complete deletion of HuR, *in vivo* is embryonic lethal.[134] Thus, a conditional knock-out of HuR in specific lung cell types in the mouse lung, such as epithelial cells and fibroblasts, would be needed to assess TGFβ1-induced differentiation and metabolic reprogramming. The effect of hypoxia could be tested simultaneously by using a hypoxic chamber for mice. An experimental model to induce fibrosis, such as bleomycin (BLM) could also be considered. Recent literature has shown that mice treated with BLM and hypoxia have worsened lung inflammation and fibrosis.[135] While the BLM model is widely-used to study fibrosis, several limitations exist such as an exacerbated inflammatory response and the inability to reproduce a slow and irreversible progression of fibrosis.[136] From a clinical perspective, it would be important to study HuR cytoplasmic localization and HuR protein levels in IPF lung tissue cells compared to normal subjects. This strategy may be useful in highlighting HuR as a biomarker for susceptibility to IPF. With continual research we may uncover a vital link in the relationship between the HuR pathway and fibrotic development in humans. This may highlight a crucial molecular mechanism that could be targeted by novel therapies based upon slowing HuR function to reduce advancement of lung fibrosis.

Conclusion

In conclusion, we are the first to report cytoplasmic translocation of HuR in human lung fibroblasts in response to hypoxia, TGFβ1 and hypoxia combined with TGFβ1. In addition, our findings show that HuR plays a role in TGFβ1-induced differentiation as the knock-down of HuR

results in a significantly decreased expression of α-SMA and collagen I. We also show that hypoxia abrogates TGFβ1-induced differentiation and metabolic reprogramming as levels of α-SMA, collagen I and lactate secretion were all diminished. Further molecular investigation is needed to study the pathway by which HuR could be acting through as well as any post-translational modifications that may be influencing its role in IPF. This important research may contribute to the development of a novel therapeutic target in the treatment of IPF and other fibrotic disorders.

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