The role of human antigen R (HuR) in myofibroblast differentiation: implications for pulmonary fibrosis

Fatmah Fahad Alhabeeb

Faculty of Medicine

Division of Experimental Medicine, Meakins-Christie Laboratories

McGill University, Montreal

2018

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

TABLE OF CONTENTS

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is an under-diagnosed lung disease characterized by progressive lung scarring with median survival of 3-5 years from initial diagnosis. Although the etiology of IPF is unclear, excessive extracellular matrix (ECM) deposition is a key event in disease pathogenesis. ECM -particularly collagen and fibronectin- is excessively deposited due to increased differentiation of fibroblasts into the α-SMA-expressing myofibroblasts by transforming growth factor (TGF) β1. We predict that TGFβ1 promotes fibrosis via human antigen R (HuR), an RNA binding protein whose principle function is to promote protein translation. HuR is localized in the nucleus under normal conditions, but upon translocation from the nucleus to the cytoplasm, HuR may promote the translation of pro-fibrotic ECM mRNA (including TGFβ1) into protein. The role of HuR in the differentiation of fibroblasts to myofibroblasts in association with ECM deposition is completely unknown. Therefore, we hypothesize that HuR promotes fibrosis by inducing the differentiation of fibroblasts to myofibroblasts and increasing ECM deposition. The aims of this study are: (1) Determine whether HuR regulates the differentiation of fibroblasts into myofibroblasts; and (2) Evaluate potential HuR mRNA targets implicated in the fibrotic process. To first explore the role of HuR in fibroblast differentiation, human lung fibroblasts (HLFs), were treated with TGFβ1 (5 μg/ml) and the mRNA and protein expression of HuR as well as the fibrogenic (α-SMA(*ACTA2*), collagen (*COL1A1* and *COL3A1*) and fibronectin (*FN1*)) markers were evaluated by qRT-PCR and western blot, respectively. Exposure of HLFs to TGFβ1 increased expression of α-SMA, collagen and fibronectin but there was no change in HuR levels. Next, cytoplasmic and nuclear protein fractions were obtained from TGFβ1-treated HFLs to assess HuR localization by western blot. In addition, immunofluorescence (IF) was also used to assess cellular localization of HuR in response to TGFβ1. There was a significant increase in translocation of HuR from the nucleus to the cytoplasm- a feature consistent with

HuR activation. To now confirm whether HuR was necessary to promote fibroblast differentiation, HLFs were transfected with HuR siRNA (siHuR) or control (scramble) siRNA (siCtrl), treated with TGFβ1 and fibrotic markers assessed as above. These results showed that siHuR-transfected cells had a significant reduction in α-SMA (*ACTA2*) expression, but no change on collagen I (*COL1A1*), III (*COL3A1*) or fibronectin (*FN1*) mRNA. Next, Actinomycin D (ActD)-chase experiments were performed to examine if HuR affects the stability of these transcripts. HuR knock-down did not affect mRNA stability of *ACTA2, COL1A1, COL3A1 or FN1*. Finally, RNA-immunoprecipitation (RIP) was performed to assess binding of HuR to the mRNA of *ACTA2*, *COL1A1*, *COL3A1* and *FN1* mRNA. These results show that in response to TGFβ1, there is enrichment of HuR binding to *ACTA2*, *COL1A1*, *COL3A1* but not *FN1* mRNA. This study is the first to investigate the role of HuR in fibroblast differentiation to myofibroblasts and consequent production of ECM. Data provided herein sets the stage for further studies aimed at investigating the role of HuR in the pathogenesis of IPF. Research on HuR could assist in establishing the basis for the development of new target therapy for fibrotic diseases such as IPF.

RESUME

La fibrose pulmonaire idiopathique (FPI) est une lésion des poumons de cause inconnue. C'est une maladie très sous-diagnostiquée avec une survie médiane de 3 à 5 ans après le diagnostic. FPI est provoquée par une cicatrisation anormale dans les poumons causant un dépôt excessif de matrice extracellulaire (MCE) dans l'interstitium pulmonaire. Le dépôt excessif de collagène et fibronectine se produit grâce à la différenciation accrue des fibroblastes en myofibroblastes. Ces dernières expriment la protéine a-SMA (pour alpha smooth muscle actin) sous l'influence du facteur de croissance transformant beta 1 (TGFβ1). Nous suggérons que TGFβ1 favorise la fibrose pulmonaire via une protéine nommée HuR (pour human antigen R). HuR une protéine de liaison à l'ARN fonctionnellement impliquées dans le transport, stabilité et traduction des ARN messagers (ARNm). Dans les conditions normales HuR est localisé dans le noyau. Cependant, en présence d'un stimulus, HuR se transloque vers le cytoplasme pour favoriser la traduction des ARNm en protéines, y compris les ARNm pro-fibrotiques (e.g. TGFβ1). Le rôle de HuR dans la différenciation des fibroblastes en myofibroblastes et dans le dépôt de MCE est complètement inconnu. Les objectifs de cette étude sont: (1) Déterminer si HuR régule la différenciation des fibroblastes en myofibroblastes; et (2) Évaluer les cibles potentielles (ARNm) de HuR impliquées dans les processus fibrotiques.

Pour répondre à la première question, des fibroblastes pulmonaires humains (HLFs), ont été traités avec TGFβ1 (5 μg/ml) suivi par la mesure du niveau d'expression de gènes HuR, α-SMA, collagène (COL1A1 et COL3A1) et

fibronectine (FN1). Nous avons observé par PCR quantitative (qPCR) et par western blot que le traitement des cellules HLFs par TGFβ1 augmentait l'expression d'α-SMA, collagène et fibronectine. Cependant, TGFβ1 n'avait pas d'impact significatif sur le niveau d'expression de HuR. Ensuite, nous avons montré par immunofluorescence que TGFßl induit la translocation de HuR vers le cytoplasme - une caractéristique indicative de son activation. Pour vérifier maintenant si HuR est nécessairepour la différenciation des fibroblastes en myofibroblastes les HLFs ont été transfectées avec des petits ARN interférents (pARNi) dirigés contre HuR (siHuR) suivi par traitement avec TGFβ1. Nos résultats ont montré que les cellules transfectées avec siHuR expriment moins d'α-SMA en comparant avec les cellules transfectées avec pARNi control (pictrl). Cependant, HuR avait peu d'effet sur le niveau d'expression de COL1A1, COL3A1 et fibronectine. Ensuite, les expériences de stabilité ont montré HuR ne semble pas réguler la stabilité de ces ARNm. Dans le but de répondre à la deuxième question, nous avons réalisé une expérience d'immunoprécipitation de l'ARN (RIP). Nos résultats ont montré que le traitement des HLFs par TGFβ1 enrichie la liaison de HuR aux ARNm d'α-SMA, COL1A1, COL3A1. Cependant, TGFβ1 n'induisait pas la liaison de HuR sur l'ARNm de FN1. Cette étude est la première à étudier le rôle de HuR dans la différenciation des fibroblastes en myofibroblastes et la production de matrice extracellulaire. Ces résultats constituent la base pour d'autres études visant à étudier le rôle de HuR dans la

pathogenèse de l'IPF. Ceci pourrait contribuer au développement d'une nouvelle stratégie thérapeutique ciblant les maladies fibrotiques ; telles que la FPI.

ACKNOWLEDGEMENT

Each creative has an achievement and each success deserves an appreciation. We must always thank and appreciate those who have helped us when we needed them the most, those who stood by us, and we must always tell them our joy in their presence and our appreciation for their support.

And as ***Frederick Koenig*** once mentioned “happiness comes not from getting something we lack, but from the recognition and appreciation of what we do have”. I would like to express my sincere gratitude to my supervisor **Dr. Carolyn Baglole**, for her support and guidance. Her continuous faith in me, motivation to pursue my research journey, a journey that I dreamed about but didn’t think I will be able to achieve. I would also like to thank my colleagues in Dr. Baglole’s lab for their contribution and creating a great environment in the lab: most importantly, Hussain Trablousi and Noof Aloufi. As well as, Necola Guerrina, Swati Parek, Kashmira Prasade, Angela Rico de Souza and Joshua Trivlidis. Thank you for the long term working rapport you have shared with me. I would also like to acknowledge the faculty members of the Meakins-Christie Laboratories, particularly Dr. David Eidelman for his invaluable scientific advice. Great thanks also to Dr. Imed Gallouzi for giving me the chance to train and practice in his lab. Dr. Sabah Hussain and Dr. Inga Murawski, thank you for your continuous guidance and help during thesis writing. Dr. Elizabeth Fixman, my Academic advisor, thank you so much for your patience, support and understanding. I would like to thank King Saud University for sponsoring my studies and giving me the chance to pursue my dream in getting my master’s degree and practice in Canada. Finally, and most importantly who has the credit for my scientific and practical journey and who also have credit for what I have reached today are my family; my mother and father, my husband and my kids and my brothers and sisters, whom I will never

be able to thank them enough for their continuous patience and support and without them I won't be the same person I am today.

CONTRIBUTION OF CO-AUTHRS

This thesis is based on my original work and is presented as a manuscript currently in preparation for peer-review and submission.

1- The human lung samples that were used in this project were provided from **Dr. Parameswaran Nair** in the Department of Medicine, McMaster University.

2- **Dr. Ilan Azuelous:** provided assistance with experimental design and with manuscript editing.

3- **Dr. Carolyn Baglole:** provided intellectual support for planning and designing experiments, in addition to editing my thesis.

CHAPTER 1: INTRODUCTION

1.1 Interstitial lung disease (ILD) and idiopathic pulmonary fibrosis (IPF):

1.1.1 Interstitial lung disease definition:

Interstitial lung disease (ILD) refers to a heterogeneous collection of more than 200 lung disorders that are characterized by varying degrees of fibrosis and inflammation of the lung parenchyma or lung interstitium [1, 2]. ILDs tend to be grouped together because they share clinical, radiological and pathological features [2]. ILDs are divided into those with known causes such as drugs and certain occupational/enviromental exposures, and those with an unknown causes, which includes idiopathic interstitial pneumonias (IIP) such as idiopathic pulmonary fibrosis (IPF) [3]. While many forms of ILD are extremely rare, others- such as IPF- are more common [4]. Among the various forms of ILD, IPF has received the most attention because of its poor prognosis and unresponsiveness to traditional therapies [1].

1.1.2 Idiopathic pulmonary fibrosis definition, epidemiology, and risk factors:

IPF is a chronic fibrosing ILD of unknown etiology [5, 6] that occurs in adults. IPF is a progressive and irreversible disease that is limited to the lungs [7]. It is characterized by the histopathological and/or radiological pattern of usual interstitial pneumonia (UIP) [7, 8]. Unfortunately, IPF is associated with extremely poor prognosis, with a median survival of only 3-5 years from diagnosis [6]. IPF is the most common of the idiopathic interstitial pneumonias (IIP) and one of the most common forms of ILD overall, accounting for 47%-71% of all IIP cases [6]. Despite this, IPF is classified as an orphan lung disease, largely due to under-reporting [6]. Due to varying case definitions, region of the lung and the methodology used for evaluation, the exact incidence and prevalence of IPF is not well-reported [6, 8]. For example, it is still unknown whether the incidence and prevalence of IPF are influenced by ethnic, racial or geographical factors. However, an increase in IPF

prevalence has recently been observed, which may reflect the advancement of diagnostic approaches, early reporting and/or increase in disease awareness [9].

IPF typically affects adults over 50 years of age and usually presents in the sixth or seventh decade of life, where the prevalence of IPF rises dramatically [7, 8]. IPF occurs more frequently in men than women, with an estimated overall prevalence of 50 cases per 100,000 people worldwide; among which 13 cases per 100,000 for females and 20 cases per 100,000 for males [4, 6]. Prevalence and incidence in Europe and USA for IPF range from 1 - 23 cases per 100,000 and 4.6 - 7.4 cases per 100,000, respectively [4, 7]. In Canada, IPF has an estimated prevalence and an incidence at approximately 41 cases per 100,000 and 18 cases per 100,000, respectively [10].

Although the cause of IPF is unknown, risk factors that either affect disease onset and/or disease progression include:

- Environmental factors: Smoking (>20 packs/year) is considered the most important risk factor, with up to 70% of IPF patients having a smoking history [7]. Occupational/environmental exposures, including exposure to silica, brass, steel, lead and wood dust, farming/agricultural work and the construction of wooden houses have also been reported to be risk factors associated with IPF [8].
- Gastroesophageal reflux (GERD): Several studies have shown that gastroesophageal reflux affects disease progression, via micro-aspirations [1, 8]. Although an association between GERD and IPF is confirmed, the cause-effect relationship is still unclear [11].
- Viral infections: viral infections (hepatitis C virus, herpes virus, adenovirus) are etiological risk factors for IPF, and may play a role in disease progression [1, 8]. Clinical and experimental evidence suggests that viruses may play a role either by predisposing to or exacerbate an existing IPF [12].

- Autoimmune disease: An autoimmune origin for IPF is based on the fact that radiologic and/or histologic manifestations of lung fibrosis are associated with connective tissue diseases, although these usually present with the histological pattern of non-specific interstitial pneumonia (NSIP). In addition to, many patients with IPF have circulating auto-antibodies, such as rheumatoid factor and complement-fixing antibodies [13]. However, the exact roles of these autoantibodies on IPF disease onset and progression remain unknown [1].

1.1.3 Idiopathic pulmonary fibrosis clinical features, natural history of the disease and treatments:

The clinical presentation of IPF is usually characterized by non-specific respiratory symptoms such as progressive dyspnea (breathlessness) on exertion accompanied by non-productive cough [14]. The gross appearance of IPF lungs by chest high resolution computed tomography shows a characteristic fibrosis (honeycombing) that is distributed along the inferior portions of the lobes with subpleural accentuation (Figure 1) [15]. Symptom onset is slow but worsens over time, with eventual respiratory failure. The presence of non-respiratory symptoms/signs should lead to suspicion of an alternative diagnosis. Currently, there is no specific test to aid in the diagnosis of this disease [14].

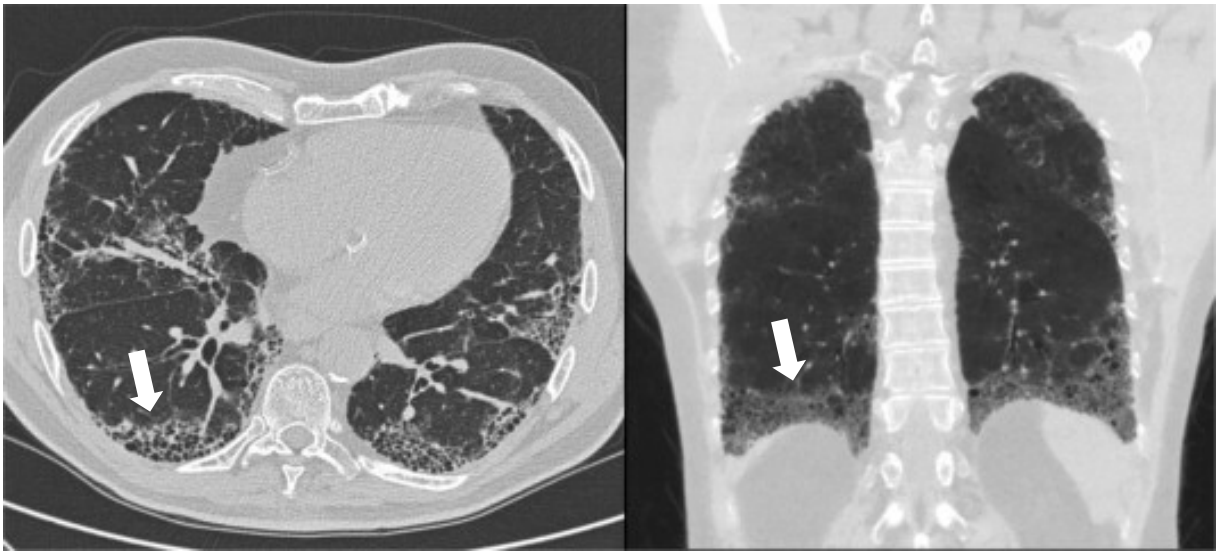

Figure 1. High resolution computed tomography (CT) of the chest showing classic UIP pattern in IPF. In both pictures, there are classic features including peripheral and basilar honeycombing and fibrotic changes (*arrows)* [16].

The typical microscopic appearance of IPF has been termed UIP and exhibits spatial (or geographic) and temporal heterogeneity [15]. A characteristic finding of UIP is the presence of fibroblastic foci at the interface between the fibrotic as well as less-involved regions of the lobule [6, 8]. These fibroblastic foci manifest as proliferations of spindled fibroblasts and myofibroblasts, often arranged parallel to the alveolar surface (Figure 2) [6, 8].

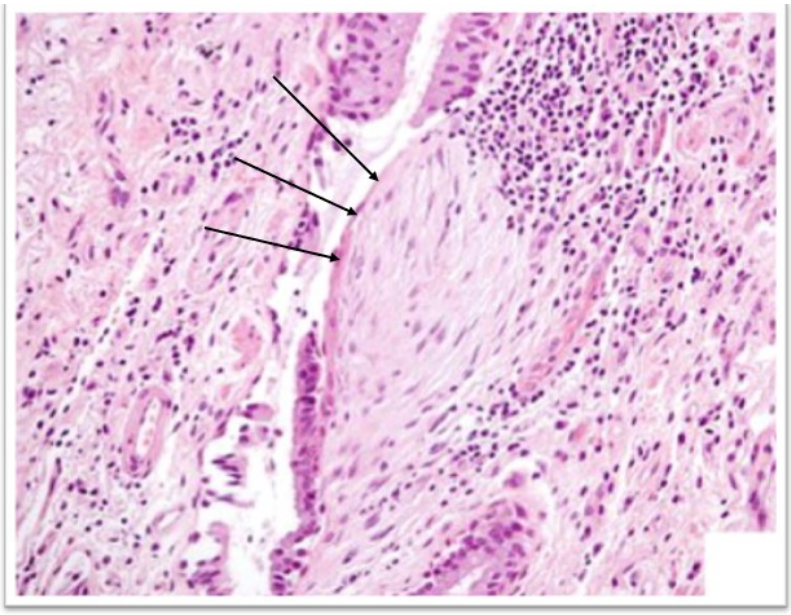

Figure 2. UIP pattern in a lung biopsy that illustrates a fibroblastic focus (*arrows*). The fibroblastic focus consists of spindle shaped cells- fibroblasts and myofibroblasts- overlying hyperplastic alveolar cells (pneumocytes). (hematoxylin and eosin stain; original magnification, ×200) [17]

The natural history of IPF is highly variable [8, 18]. Some patients remain asymptomatic for 2-3 years and only present after an extensive damage to the lungs [18]. However, most patients have slow progression with clinical and functional deterioration that eventually results in chronic respiratory failure [6, 8]. For others, there are periods of relative stability intermixed with episodes of acute worsening (*i.e.* acute exacerbations or other complications, such as; heart failure), which are a cause of high morbidity and mortality [6, 8]. In a minority of patients, there is rapid progression (accelerated form) with rapid progression and even death within 6 months from diagnosis [7]. In general, the average survival is 2–5 years from symptom onset [7, 8]. It is unknown whether the different forms of this natural history represent different disease phenotypes [8].

Thus, IPF is a considerable challenge for clinicians. There is no known medical therapy that can enhance survival and/ or improve outcomes for patients with IPF. The only two pharmacological anti-fibrotic therapies approved for IPF are Pirfenidone and Nintedanib [19]. These drugs slow fibrosis progression but unfortunately do not improve survival [19]. Lung transplantation is currently the only option that can provide significant long-term

survival, however, only a small proportion of IPF patients are eligible to receive transplantation [20]. Notably, traditional therapy for ILD that use corticosteroids or other immunomodulatory drugs have proven ineffective. As such, current drug development is focused on targeting fibrogenesis and fibroproliferation, and an increasing number of targeted therapies are currently in clinical trial [20]. Significant research has been conducted over the past decade in the hope of having a better understanding of the disease pathogenesis to find a treatment that can slow disease progression and improve survival. The following section will elaborate on disease pathogenesis and the cell types involved in the fibrotic process.

1.2 IPF pathogenesis:

1.2.1 General overview of IPF pathogenesis:

Although the exact pathophysiological mechanism for IPF is still unknown, experimental and clinical research has focused on identifying the cellular and molecular mechanisms that contribute to the development of IPF [8]. Previous views that inflammation was essential for IPF development have been replaced by the concept that IPF arises as a consequence of impaired alveolar wound healing [21]. The shift in paradigm that IPF is not an inflammatory disease is largely based on (1) presence of minimal signs of inflammation on lung biopsy and (2) ineffective response to immunosuppressive and corticosteroid therapies in treating IPF. As such, the rejection of the "inflammation-driven fibrosis" hypothesis has led to the "fibrogenesis" theory [21].

The sequence of events for fibrogenesis can be subdivided into three pathophysiologic stages. Stage 1 is the predisposition stage, whereby an unknown genetic mutation(s) predisposes an individual to develop lung fibrosis. Stage 2 is followed by chronic epithelial cell turnover in response to injury, which is coupled with environmental exposure to risk

factors such as cigarette smoke or occupational/environmental exposures [1]. Collectively, these events lead to epithelial cell dysfunction and the development of an aberrant basement membrane. Not all individuals in this stage will necessarily develop clinically-relevant disease; whether they do or do not depends on the degree and duration of exposure to the aforementioned risk factors [8].

The most clinically-relevant genetic alterations in IPF are mutations in genes that maintain the length of the telomeres (TERT (Telomerase reverse transcriptase), TERC (telomerase RNA)), which – although rare - are more common in the familial forms of IPF. Studies have shown that telomere shortening could promote the loss of alveolar epithelial cells, resulting in aberrant epithelial cell repair, and should be considered as a contributing factor for IPF. Additionally, dysregulated expression of surfactant protein C (SPC) and the mucin 5B promoter region (MUC5B) have been reported as well in familial and sporadic IPF cases [22, 23]. There are currently no established genetic tests to assess predisposition to IPF [22].

The development of IPF is thought to be ultimately due to repetitive microscopic alveolar epithelial cell injury and dysregulated repair, followed by fibrosis and excessive deposition of extracellular matrix (ECM). Ultimately, these events result in the loss of parenchymal architecture and lung function [24]. Initial alveolar epithelial type II cell damage by microinjuries and subsequent disruption of the continuity of the basal lamina within the alveoli is considered to be one of the key trigger mechanisms [24]. Consequently, released profibrotic factors, particularly transforming growth factor-β1 (TGFβ1), lead to recruitment, proliferation and differentiation of fibroblasts into α-smooth muscle actin (α-SMA)-expressing myofibroblasts. Myofibroblasts are the key effector cells in IPF that produce copious amounts of ECM proteins. The deposition of ECM is associated with the formation of typical fibroblastic foci. Myofibroblasts that accumulate in fibroblastic foci are

considered the hallmark cells in the development of lung fibrosis. The origin of myofibroblasts in pulmonary fibrosis is still controversial, [25] but may include epithelial–mesenchymal transition (EMT), fibrocyte recruitment, pericyte trans-differentiation, pleural mesothelial cells or expansion of the resident lung fibroblast population [26]. Apoptosis of the fibroblasts and myofibroblasts in the lungs of patients with IPF is decreases but in alveolar epithelial cells is increased, resulting in impaired re-epithelialisation and restoration of the normal lung architecture, leading to continuous and extensive ECM deposition (Figure 3) [26].

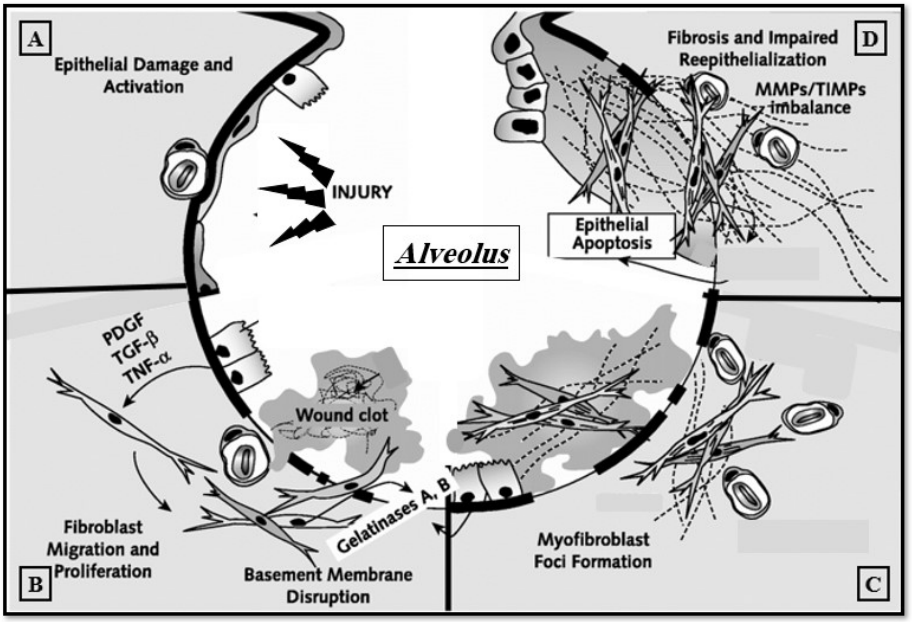

Figure 3. Pathogenesis of IPF. *Top left panel (A)*: microinjuries damage and activate alveolar epithelial cells, which secrets growth factors, including TGFβ1, to induce migration, proliferation and differentiation of fibroblasts to myofibroblasts. *Bottom left panel (B)*: Extensive injury to alveolar epithelial cells increases basement membrane disruption and allow further fibroblast migration. *Bottom right panel (C)*: accumulated fibroblast-myofibroblast will secrete ECM proteins. *Top right panel (D)*: there is an imbalance in matrix deposition and tissue inhibitors of matrix metalloproteinases (TIMPs) that lead to progressive unopposed matrix deposition and development of IPF (adapted from [27]).

Numerous pro-fibrotic mediators, including interleukin 1β (IL-1β), tumor necrosis factor-α (TNFα), connective tissue growth factor (CTGF), TGFβ1 and platelet-derived growth factor (PDGF), are involved in pulmonary fibrosis. Many of these mediators are responsible for chemotaxis and differentiation of myofibroblasts. The signaling cascades governed by these mediators also play an important role in the pathogenesis of fibrotic lung diseases [21]. For example, TGFβ1, one of the main pro-fibrotic cytokines in IPF, has multiple functions, including promoting chemotaxis and proliferation of fibroblasts, differentiation of fibroblasts into myofibroblasts, EMT and protection of myofibroblasts from apoptosis. TGFβ promotes the production of additional pro-fibrotic cytokines and tissue inhibitors of metalloproteinases (TIMPs) in addition to inhibiting matrix-degrading proteases [26]. Additionally, vascular endothelial growth factor (VEGF) stimulates (neo)angiogenesis and is increased in capillary endothelial cells and alveolar epithelial cells (AEC) II in IPF patients [28]. The following sections will focus on the cells involved in fibrosis as well as the cytokines and mediators that are implicated in proliferation, migration and trans-differentiation of the fibroblast into myofibroblasts.

1.2.2 Cells involved in the fibrotic process:

1.2.2.1 Fibroblasts:

Epithelial cell dysfunction and abnormal basement membrane lead to the activation of fibroblasts that culminates in deposition and remodeling of the ECM. Fibroblast activation in turn leads to pro-fibrotic changes in lung fibroblasts. The myofibroblast is the classic pathologic fibroblast phenotype in IPF lungs [29]. Additional phenotypic changes of fibroblasts that could contribute to the development of lung fibrosis include resistance to apoptosis [30]. During normal wound healing, fibroblasts are removed by apoptosis. This clearance mechanism limits ongoing matrix deposition and the development of fibrosis [31].

Unlike normal fibroblasts, IPF fibroblasts resist apoptosis and have greater proliferative capacity [30]. Activation of pro-survival pathways in IPF fibroblasts may lead to their retention in IPF lungs, allowing fibroblasts to continuously deposit collagen; this ultimately leads to pathologic lung remodeling. In addition, the anatomic appearance of these cells suggests that IPF fibroblasts can also invade the ECM more readily than non-IPF fibroblasts [32]. The mechanisms for this enhanced invasion are poorly understood, but this invasive property correlates with levels of α- smooth muscle actin (α-SMA) expression [30].

1.2.2.2 Myofibroblasts:

Myofibroblasts are essential for wound healing, participate in tissue remodeling following insult and are the major cell type within fibroblastic foci. Myofibroblasts are largely activated fibroblasts, although they can be derived from epithelial, endothelial or mesenchymal cells [33]. Under the direction of cytokines such as TGFβ1, PDGF and CTGF, fibroblasts differentiate into myofibroblasts [34]. In addition to their role in wound healing, myofibroblasts contribute to fibrotic processes and tumor invasiveness. Myofibroblasts are important in the normal lung architecture, as they deposit and maintain ECM proteins such as collagens (I, III, IV, V and VI), glycoproteins and fibronectin. Myofibroblasts also acquire cytoskeletal characteristics of contractile smooth muscle cells via the expression alpha-SMA, a feature that also distinguishes myofibroblasts from fibroblasts. Compared with resident lung fibroblasts, myofibroblasts secrete excessive amounts of matrix components, including type I collagen. This excess matrix deposition may lead to pathologic lung fibrosis and remodeling [33].

1.2.2.3 Alveolar epithelial cells:

There are two types of epithelial cells in the alveolar wall: type I AEC are thin and flat and form the structure of the alveoli whereas type II AECs secrete surfactant [35]. It is possible that EMT contributes to the resident pool of myofibroblasts in IPF. EMT is the process by which epithelial cells acquire features commonly associated with mesenchymal cells following activation by growth factors such as TGFβ [36]. EMT is associated with changes in protein expression whereby epithelial cells lose their polarity and tight junctions and become more mobile. EMT can occur during development, cancer and fibrosis. Although evidence indicates that epithelial cells acquire mesenchymal features in IPF lungs, the contribution of these mesenchymal changes to the fibrotic process remain unresolved [26]. One ongoing controversy is whether epithelial cells acquire sufficient mesenchymal characteristics that they can be classified as fibroblasts. In humans, the only data supporting this possibility is the finding that fibroblasts isolated from IPF patients express the epithelial cell surface marker N-cadherin [37].

1.2.2.4 Fibrocytes:

Fibrocytes are spindle-shaped, circulating mesenchymal cells that have the ability to differentiate into myofibroblasts, osteoblasts and chondrocytes [38]. Fibrocytes are believed to be involved in the pathogenesis of several fibrotic disorders affecting lungs, liver and kidneys, but their exact biological role is not fully understood [38]. Fibrocytes respond to tissue-derived signals and migrate to sites of injury where they can differentiate into fibroblast-like cells capable of producing ECM proteins, enzymes, cytokines and growth factors [39]. Fibrocytes respond to the profibrotic cytokine TGFβ1 by expressing α-SMA, which supports a role for fibrocytes in myofibroblast formation [40]. The most commonly-used marker to identify fibrocytes is the surface expression of CD45 [38, 41]. Studies have

demonstrated that there are increased amounts of fibrocytes in the lungs and circulation of IPF patients, suggesting they may participate in fibrosis [42]. A role for fibrocytes was shown in radiation- and bleomycin-induced animal models, whereby the level of fibrocytes correlated with the level of fibrosis as well as collagen deposition in mouse lungs. In IPF patients, there is a 6-10% increase of circulating fibrocytes [43, 44]. As such, it has been proposed that fibrocytes contribute to fibrosis via differentiation into CD45-negative (myo)fibroblasts that both secrete ECM and are a putative source for the fibroblastic foci [41].

1.2.3 Cytokines and growth factors:

1.2.3.1 TGFβ and other growth factors:

The TGFβ subfamily, composed of three isoforms (TGFβ1, 2 and 3), are multifunctional cytokines that play a central role in wound healing, tissue repair as well as other important cellular functions [45]. TGFβ1 is expressed in all tissues, but is particularly abundant in bone, lung, kidney and placenta [46]. TGFβ1 is produced by most parenchymal cells as well as by immune cells such as lymphocytes, monocytes/macrophages and platelets [47]. Under normal conditions, inactive TGFβ1 is synthesized and secreted bound to the latency-associated peptide (LAP) and stored in the interstitium [45]. However, during injury, TGFβ1 is released [48]. Here, the latent form of TGFβ1 is activated in IPF via integrins, specifically αvβ6. Active TGFβ1 signals via the canonical SMAD (homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the *Caenorhabditis elegans* protein Sma)-related pathways as well as an emerging array of non-canonical SMAD-independent mechanisms [49]. TGFβ1 first binds to the type II receptor (TβRII), which is expressed in the cell membrane in an oligomeric form with intrinsic kinase activity; TGFβ type I receptor (TβRI) is then recruited and phosphorylated by TβRII, leading to activation of

its kinase activity and subsequent intracellular signaling [50]. TGFβ-receptor activation enables the association of TGFβ1-receptor complex with R-Smads (receptor-regulated-SMAD2/3) with co-Smad (common-SMAD4) and, consequently, to regulate gene expression in the nucleus [51].

Levels of active TGFβ1 are increased in the lungs of patients with IPF [52]. An increase in ECM deposition was associated with TGFβ1 activation in fibrotic tissue [53]. Furthermore, inhibition of AEC proliferation, apoptosis, EMT, ECM production and differentiation of fibroblasts to myofibroblasts all are a consequence of excessive TGFβ1 signaling in IPF [48].

It is now readily apparent from current IPF research that the mechanisms driving fibrosis reflect abnormal, dysregulated wound healing within the lung, involving increased activity and exaggerated responses by a spectrum of pro-fibrogenic growth factors. In addition to TGFβ1, PDGF, CTGF, PDGF and VEGF also play a role in pulmonary fibrosis [54]. Despite the fact that TGFβ1 is the primary cytokine in the fibrogenic process in IPF, the other growth factors support fibro-pathogenesis through recruitment of fibroblasts, secreting ECM, angiogenesis and fibroblast differentiation and proliferation [55]. For example, PDGF is a potent fibroblast mitogen and chemoattractant. In normal adult lung, PDGF and PDGFR (Platelet-derived growth factor receptor) are expressed at low levels in alveolar macrophages, but they are upregulated in IPF. Additionally, in early-stage IPF, type II AECs and mesothelial cells express PDGF and PDGFR. On the other hand, CTGF is a potent enhancer of fibroblast proliferation, chemotaxis and ECM deposition. In mesenchymal cell types, CTGF induction is primarily, but not exclusively, mediated by TGFβ through a TGFβ-response element in the *CTGF* promoter. In the lung, CTGF is secreted from multiple sources, such as fibroblasts and bronchial epithelial cells. Many recent studies on IPF patients have shown an increased expression of CTGF to be associated with fibroproliferative

disorders. However, the exact mechanism of this is still not understood. It is still not known whether the previously mentioned growth factors are essential for the development of fibrosis [54].

1.2.4 Molecular regulation in IPF:

Despite extensive research on IPF, the exact pathogenesis is not clear. Emerging topics of interest include epigenetic regulation of gene expression transcriptionally at the level of the DNA and post- transcriptionally at the level of RNA and protein. Post-transcriptional regulation is a highly conserved process that contributes to normal development and adaptation to changes in cellular and organ homeostasis. Evidence for the role of the post-transcriptional regulation in the development of IPF is based on studies demonstrating an association of specific markers relevant to the fibrotic cascade. Post-transcriptional regulation often involves stabilization of mRNAs. The half-life of an mRNA can be altered by microRNAs (miRNA) and RNA-binding proteins (RBP). miRNA-154 is an example of a miRNA that is up-regulated in IPF and induced by TGFβ1 in human lung fibroblasts. miRNA-154 induces cell proliferation and migration of fibroblasts [56]. miRNA-21 is another miRNA up-regulated in IPF and induced by TGFβ1 in lung fibroblasts from IPF patients [57]. On the other hand, IGFBP-5 (insulin-like growth-factor-binding protein (IGFBP)-5) is an example of an RNA-binding protein up-regulated in primary skin and lung fibroblasts cultured from fibrotic areas in patients with systemic sclerosis. IGFBP-5 induces collagen and fibronectin production from fibroblasts and induces fibroblast/myofibroblast differentiation *in vitro* and *in vivo* [58].

HuR (Human Antigen R) is another example of a RNA-binding protein that was found to be up-regulated in cardiac fibroblasts treated with the profibrotic stimulus TGFβ1. HuR levels were correlated with the level of cardiac fibrosis in *in vivo* models [59]. However,

the role of HuR in IPF has never been examined. Targeting post-transcriptional regulation by these miRNAs and RNA-binding proteins directly or indirectly may promote/attenuate fibrosis and hence, IPF.

1.3 Human antigen R (HuR):

1.3.1. Post-translational regulation by HuR:

Post-transcriptional regulation of gene expression includes mRNA transportation, stability, translation and degradation [60-64]. Two main factors that govern post-transcriptional regulation are the turnover and translation of regulatory RNA-binding proteins (TTR-RBP) and non-coding RNA (ncRNA) [64-66]. Each subgroup of TTR-RBP and ncRNA has specific functions that determine the fate of mRNA [60, 62]. One of the best characterized RBPs is encoded by the *embryonic lethal, abnormal vision-like 1* (*ELAVL1*) gene. Members of ELAV family of proteins control the fate of mRNA by inducing stability, translocation and translation of their targets mRNA [60, 67, 68]. Other members of this family include HuB, HuC and HuD, which are specifically expressed in the nervous system. In contrast to other RBPs in the *ELAVL1* family, HuR is ubiquitously-expressed [64, 67, 69]. HuR participates in the post-transcriptional regulation of mRNA and is one of the best-described regulators of mRNA fate in the cell [60, 69, 70]. HuR exerts its effect on cellular functions when it binds to its target mRNA in the nucleus, [67, 71] specifically to adenosine -and- uridine rich elements (ARE's) in the 3' untranslated region (UTR) [70, 72, 73]. These ARE sequences are involved in mRNA stability and are found in oncogenes, proto-oncogenes, cytokines, chemokines and growth factors that influence cell growth, angiogenesis and metastasis [74, 75]. For example, the majority of chemokine and cytokine mRNAs contain AREs [61].

1.3.2 Molecular structure and cell location:

In a resting cell, HuR is predominantly localized in the nucleus. Upon activation by stress conditions such as ultraviolet (UV) radiation, proliferation, nutrient depletion or immune activation, HuR binds to target mRNA and shuttles to the cytoplasm [70, 76]. It is believed that cytoplasmic localization is key to HuR function [73], which affects mRNA stability and/or translation by interacting with miRNAs or other RBPs including TIA-1 (T-cell intracytoplasmic antigen-1), TTP (tristetraprolin), BRF1 (butyrate response factor-1), heterogeneous nuclear ribonucleoprotein D (hnRNPD; AUF1 (AU-binding factor 1)) and KSRP (KH-type splicing regulatory protein) (Figure 4)[61].

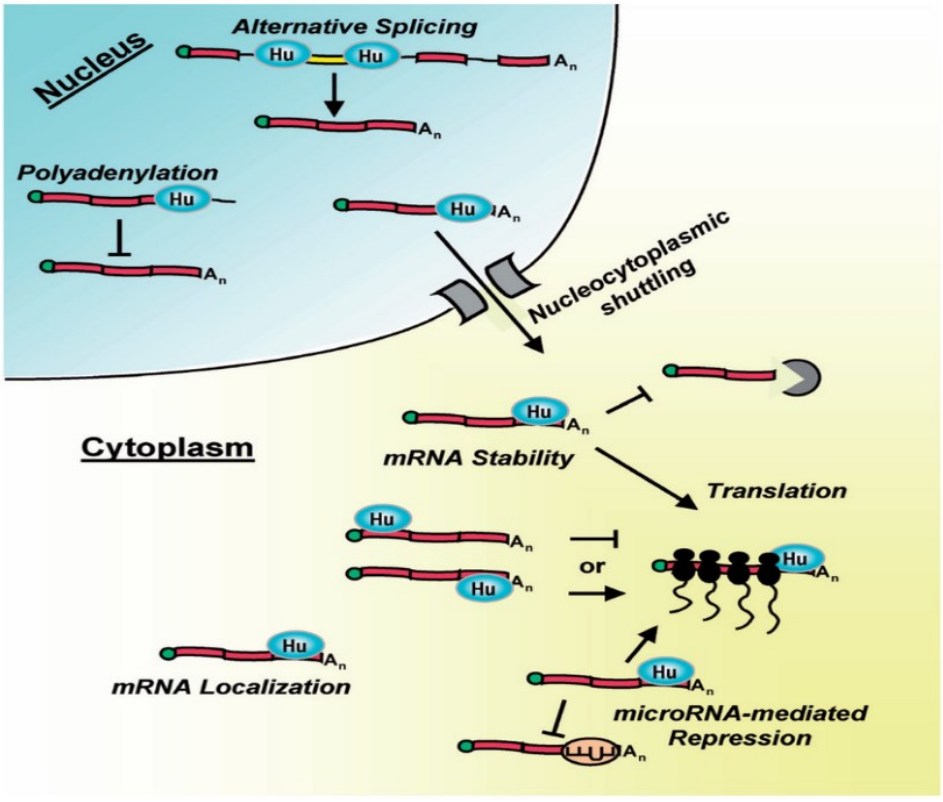

Figure 4: Diverse mechanisms of Hu protein functions. Hu proteins mediate many post-transcriptional processing events in both the nucleus and cytoplasm [69]

The structure of HuR facilitates binding to its target mRNA. HuR has three RNA-recognition motifs (RRM). The first and second RRM binds to specific RNA sequences and the third RRM binds to poly(A) tail of mRNA [68, 77, 78]. Between the second and third RRM is the hinge region which aids in HuR shuttling between the nucleus and cytoplasm through the HuR-nucleocytoplasmic shuttling (HNS) pores (Figure 5) [76, 79].

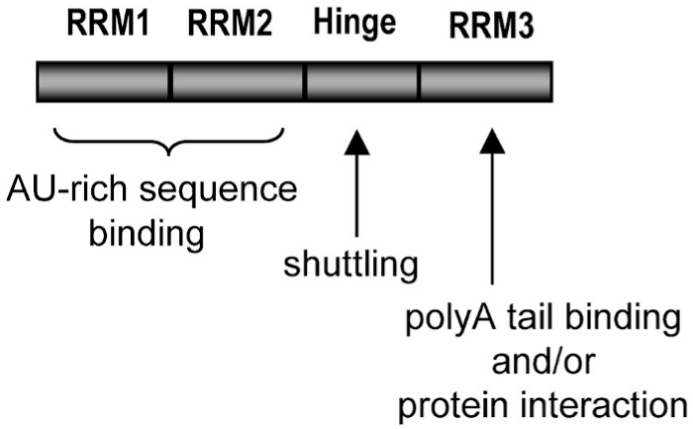

Figure 5: The HuR protein consists of 326 aa (36 kDa). HuR has three highly conserved motifs belonging to the RNA recognition motif (RRM) superfamily and a hinge region between RRMs 2 and 3 named the HuR nucleocytoplasmic shuttling (HNS) domain [69].

The ability of HuR to translocate to the cytoplasm is regulated by various mechanisms, such as HuR structure, HuR transport proteins, kinases, miRNAs and autoregulation [80]. One mechanism relies on the hinge region between the second and third RRM, supported by the presence of the HuR transport proteins (transportins 1/2) [62, 75, 81]. Additionally, HuR phosphorylation by different kinases, including checkpoint kinase 2 (Chk2), cyclin-dependent kinase 1 (Cdk1), protein kinase C (PKC), and p38 mitogen-

activated protein kinase (MAPK) is another mechanism that aids in HuR sub-cellular localization [73, 78]. Lastly, methylation and ubiquitination of HuR is accomplished by coactivator-associated arginine methyltransferase 1 (CARM1) and E3 ligase, respectively [80]. HuR translocation in the cytoplasm is inhibited by the AMP-activated protein kinase (AMPK), a ubiquitous enzyme that functions as a cellular sensor of metabolic stress and mediates HuR nuclear import through phosphorylation of the adaptor protein importin α. By promoting HuR nuclear localization, the AMPK-mediated pathway impairs its cytoplasmic effects on mRNA stability and translation [67]. Conversely, inhibition of AMPK causes a marked increase in the cytoplasmic levels of HuR, which correlates with increased HuR function [80]. Cytoplasmic function of HuR can also be affected by miRNA (e.g. miR-125a and miR-519) as these miRNA reduce HuR mRNA expression and protein and/or alter the binding of HuR with target mRNAs [73]. Not surprisingly, HuR can regulate its cytoplasmic expression as well through autoregulation, HuR binds to the HuR 3′UTR mRNA and enhances the cytoplasmic export of HuR mRNA [80].

1.3.3 Target genes and cellular process:

HuR targets mRNAs that encode proteins that plays a role in normal biological functions, including cell growth [62], survival [75], invasion, angiogenesis and inflammation [65]. Hu levels and activity in the cell is crucial for maintaining cell survival and proliferation. Whole-body deletion of HuR is embryonic lethal due to multi-organ developmental defects in the spleen, bone and lungs. [71, 82]. One of the main functions of HuR is stabilization of anti-apoptotic mRNAs targets (such as BCL2 (B-cell lymphoma), MCL1 (Induced myeloid leukemia cell differentiation protein) and p21). However, during lethal stressors (such as heat shock, staurosporine or exposure to proteasome inhibitors), HuR induces cell apoptosis- when cell death is unavoidable- by activating the apoptotic pathway

[70].

HuR function in the cell can change depending on the stimulus [69]. For instance, HuR can stabilize inducible nitric oxide synthase (iNOS), granulocyte macrophage colony stimulating factor (GM-CSF) and TNF-α [62] and prevents their decay by avoiding the binding of mRNA with other TTR-RBPs or miRNA associated with RNA-induced silencing complex (RISC) [83]. HuR can also increase the translation of certain mRNAs such as VEGF or HIF1α by facilitating their binding with the internal ribosomal entry side (IRES) [62, 69, 84].

1.3.4 HuR in disease

HuR has been implicated in a variety of pathological conditions such as cancer, fibrosis and inflammation [73]. Progress in cancer research has shed insight onto how HuR structure relates to its function. Cancers in which HuR has been implicated include renal cell, lung, ovarian, colon and liver cancers [74, 85]. For example, high levels of cytoplasmic HuR correlate with poor prognosis in cancer patients [68, 78]. High HuR expression in cancer tissue correlates with metastasis, drug resistance and poor survival. HuR is thought to stabilize and aid in translating mRNA that can lead to malignant transformation; these include mRNA encoding proteins involved in angiogenesis (VEGF and HIF1α), cell proliferation (c-MYC and cyclins), metastasis (MMP9) and pro-survival (prothymosin-α) [73]. Because of its role in stabilizing many inflammatory mRNAs [61], HuR is implicated in inflammatory-associated diseases such as rheumatoid arthritis and inflammatory bowel disease (IBD) [80]. HuR is known to stabilizes inflammatory cytokines such as cyclooxygenase-2 (COX2), IL-8 and interferon- gamma (IFNγ). Interestingly, anti-inflammatory cytokines such as IL-19 and IL-10 can repress the ability of HuR to stabilize pro-inflammatory mRNA, which explains part of their function as anti-inflammatory

mediators.[65, 86]. The aforementioned studies suggest that the HuR expression pattern might differ, possibly reflecting a different effector as well as intrinsic cellular functions.

1.3.5 Role of HuR in fibrosis:

Since the discovery of HuR in 1996, the role of HuR in diseases has been most extensively studied in diseases of inflammation, but in particular cancer. Although the role for HuR in the pathogenesis of fibrotic disease is unknown, it was recently found that HuR stabilizes TGFβ1 mRNA [87]. Moreover, there is increased cytoplasmic HuR in fibrotic processes, including during fibroblast differentiation to myofibroblasts [59, 88]. Strong evidence for a role of HuR in fibrosis comes from data where PDGF/TGFβ1-treated liver hepatocytes exhibit more fibrotic features [89], an effect that was attenuated by knockdown of HuR [90]. While HuR is emerging as a regulator of liver and cardiac fibrosis, a role for HuR in pulmonary fibroblasts is completely unknown.

CHAPTER 2: HYPOTHESIS AND AIMS

HYPOTHESIS:

HuR promotes the differentiation of fibroblasts to myofibroblasts and increases ECM deposition.

AIMS:

AIM 1. Determine whether HuR regulates the differentiation of fibroblasts into myofibroblasts in human lung fibroblasts treated with TGFβ1.

AIM 2. Determine the potential HuR-targets implicated in fibrotic process in human lung fibroblasts treated with TGFβ1

CHAPTER 3: EXPERIMENTAL PROCEDURE

3.1 Reagents:

The reagent name and the company they were purchased from will be mentioned in the corresponding methodology section as outlined below.

3.2 Subject characteristics of the human lung fibroblasts (HLFs):

HLFs used in this study were derived from lung tissue obtained from subjects undergoing lung resection surgery at McMaster University [91]. This study was approved by the Research Ethics Board of St, Joseph's Healthcare Hamilton and an informed written consent was obtained from each patient. This study was conducted on HLFs from a single normal subject with no smoking history or relevant risk factors (*e.g.* radiation or medication) for lung fibrosis.

3.3 Cell culture:

HLFs were cultured in Gibco™ Minimum Essential Media (MEM) (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) supplemented with gentamycin (WISENT Inc, Canada), Antibiotic-Antimycotic (WISENT Inc, Canada) and glutamax (Thermo Fisher Scientific, USA). Cell at passages between 4 and 11 were used for all experiments.

3.4 Western blot:

Fibroblasts were cultured with serum-free MEM for 18 hours before treatment with TGFβ1 from 12-72 hrs. HLFs were then rinsed with PBS and lysed by RIPA buffer (Thermo Scientific, Rockford) containing protease inhibitor cocktail (PIC, Roche, US). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay kit (Thermo

Fisher Scientific, USA). Cell lysates were mixed with loading buffer and boiled for 10 min. Protein samples (20 µg per lane) were electrophoresed on 7.5% SDS PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and resolved to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). After blocking for 1 h (5% non-fat dry milk in 1x PBS with 0.1% Tween 20), the membranes were incubated at 4 °C overnight with an anti-HuR antibody (1:2000; Santa Cruz, CA). On the next day, the membranes were probed with the Horseradish peroxidase (HRP)-linked anti-mouse IgG (1:10000, Cell Signaling Technologies, CA). Membranes were then visualized with Clarity™ western ECL substrate (Bio-Rad Laboratories, Mississauga, ON) or Amersham™ western ECL substrate (GE Healthcare, Italy). Protein bands were visualized using a ChemiDoc™ MP Imaging System (Bio-Rad, CA). Densitometric analysis was performed using Image Lab™ Software Version 5 (Bio-Rad, CA). Tubulin (1:50000; Sigma, CA) was used as the loading control. Protein expression was normalized to tubulin. Additional antibodies included anti-α-SMA (1:5000; Sigma, CA), anti-Col1A1 (1:200; Santa Cruz, CA), anti-Col3A1 (1:200; Santa Cruz, CA) and anti-Fn (1:200; Santa Cruz, CA).

3.5 Quantitative RT-PCR:

Total RNA was isolated with Trizol according to manufacturer's instructions (Invitrogen, USA). The concentration and quality of the RNA were then confirmed using NanoDrop 1000 spectrophotometer (Infinite M200 pro, TECAN, CA). Reverse transcription of 25 ng of RNA to cDNA was performed using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Mississauga, ON) according to the suggested protocol. cDNA was used for qRT-PCR analysis in 96-well optical reaction plates. Quantitative PCR (qPCR) was done by addition of 1 µl of cDNA and 0.5 µM primers with SsoFast™ EvaGreen® (BioRad Laboratories, Mississauga, ON). PCR amplification was performed using a CFX96 Real-

Time PCR Detection System (Bio-Rad, CA). Thermal cycling was initiated at 95°C for 3 minutes and followed by 39 cycles of denaturation at 95°C for 10 seconds and annealing at 59°C for 5 seconds. Gene expression was analyzed using the ΔΔCt method, and results are presented as fold-change normalized to housekeeping gene GAPDH. The primers were designed and purchased from Integrated DNA Technologies (Marlton, NJ). Primer sequences are in Table 2.

Table 1: primer sequences used for qRT-PCR analysis

Gene	Forward primer sequence	Reverse primer sequence
HuR	AACGCCTCCTCCGGCTGGTGC	GCGGTAGCCGTTCAGGCT GGC
α-SMA	GACCGAATGCAGAAGGAGAT	CACCGATCCAGACAGAGTATTT
COL1A1	CAGACTGGCAACCTCAAGAA	CAGTGACGCTGTAGGTGAAG
COL3A1	GCTCTGCTTCATCCCACTATTA	CTGGCTTCCAGACATCTCTATC
FN1	CTGAGACCACCATCACCATTAG	GATGGTTCTCTGGATTGGAGTC
GAPDH	GTCTCCTCTGACTTCAACAGC	ACCACCCTGTTGCTGTAGCCA

3.6 Immunofluorescence:

HLFs were treated with TGFβ1 for 6, 24 and 48 hours, then, fixed with 2 and 4% paraformaldehyde for a total of 15 min and permeabilized for 30 min in phosphate-buffered saline (PBS) containing 0.5 % Triton. After incubation with blocking buffer (Dako) for 1 hour at room temperature, cells were incubated in a 1:300 dilution of anti-HuR antibody in blocking buffer (Dako) for 2 hours at room temperature. Cells were washed with 1x PBS, incubated for 1 hour at room temperature with the secondary antibody (Alexa fluor 488, 1:1000) in antibody diluent solution (Dako). Cells were washed with PBS and nucleus was

stained with the 4'-6-diamidino-2-phenylindole (DAPI) for 15 min (1:1000). Cell images were acquired with a Zeiss LSM 780 confocal microscope (Zeiss, Oberkochen, Baden-Württemberg, Germany). ImageJ software was used to process and analyze the images for assessing HuR expression.

3.7 Cytoplasmic and nuclear protein fractions:

Cytoplasmic and nuclear protein fractions were obtained using a nuclear extraction kit as per manufacturer instructions (Active Motif, Carlsbad, CA). For these experiments, HLFs were untreated or treated with TGFβ1 (5ng/ml) for various times. In addition, cells were also treated with actinomycin D (0.5 μg/ml) for 6 hours. After treatments, the cells were resuspended in 1ml of ice-cold PBS/Phosphatase inhibitor buffer. Unlysed cells, nuclei, and cell debris were pelleted by centrifugation at 4000 xg for 5 min. Cytoplasmic and nuclear fractions were generated by washing with hypotonic buffer and complete lysis buffer, respectively. Protein concentrations were determined by the BCA protein assay kit. Western blot and antibodies used were described in Section 3.4. Lamin A/C (1:1000; Cell Signaling Technologies, CA) was used as a marker for nuclear fraction.

3.8 HuR siRNA knock-down:

Approximately 100,000 HFLs were seeded into 6-well plates containing 2 ml of 10% FBS/MEM without antibiotics and allowed to grow overnight for 24 hours. Transfections were performed with either 60 nM of HuR small interfering RNA (HuR-siRNA) or control (scrambled) siRNA (Ctrl-siRNA; Santa Cruz, CA). siRNA-transfected cells were incubated for an additional 24 hours, followed by serum starvation for 18 hours. Cells were harvested 4,

8, 24 and 48 hours after 5 ng/ml TGFβ1 (protein) or at, 3, 6, 24 and 48 hours for RNA. HuR, α-SMA and ECM expression was assessed by western blot and qRT-PCR, respectively.

3.9 Determination of mRNA stability:

HLFs transfected with control siRNA and HuR siRNA oligos, were treated with or without TGFβ1 for 24 hours. ActinomycinD (1μg/ml) was then added for an additional 1, 3 or 6 hours to block transcription [92]. Extracted mRNA was quantified by qRT-PCR. The mRNA decay was calculated as the percentage of mRNA remaining over time compared with the amount before the addition of actinomycin D. The normalized value at TGFβ1 at 0 h was set as 100%.

3.10 RNA immunoprecipitation:

RNA immunoprecipitation was used to determine whether HuR binds directly to mRNA in HLFs [93]. Briefly, HLFs were harvested and treated with TGFβ1 (5 ng/ml) for 24 hours. After treatment, cells were rinsed and then collected using 1xPBS. The HuR–RNA complexes were then immunoprecipitated using either the HuR antibody or normal mouse IgG bound with protein A-Sepharose magnetic beads (GE Healthcare, Italy). The magnetic beads were pre-coated with 15 μg of IgG (Cell Signaling Technologies, CA) or 30 μg anti-HuR (Santa Cruz Biotechnology) antibodies. The complexes underwent serial washings to wash out unbound materials. Immunoprecipitated RNAs were then extracted and analyzed by qRT-PCR. RNA expression was normalized to GAPDH mRNA bound in a non-specific manner to IgG.

3.11 Statistical analysis:

All values are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) (for multiple comparisons) and an unpaired two tailed t test to analyze the differences between two groups. A p value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc. USA). For mRNA stability, we calculated the half-lives of mRNAs on a one-phase exponential decay model. The semi-logarithmic curves were also analyzed by GraphPad Prism software. Primers sequences were listed previously (Table 1).

CHAPTER 4: RESULTS

4.1 TGFβ1 increases fibroblast differentiation into myofibroblasts: α-SMA is a marker of fibroblast differentiation into myofibroblasts. Myofibroblasts play a dominant role in pulmonary fibrosis by increasing the production of ECM. TGFβ1 is a pro-fibrotic cytokine that potently induces fibroblast differentiation. We assessed the differentiation of fibroblasts by measuring the expression of α-SMA (*ACTA2*) as well as key components of ECM including collagen isoforms (Collagen 1A1(*COL1A1*) and Collagen 3A1(*COL3A1*)) and fibronectin 1 (*FN1*) in HLFs exposed to TGFβ1 (5μg/ml) using qPCR and western blot. TGFβ1 elicited significant increase in mRNA levels of *ACTA2* at 24 and 48 hrs as well as those of *COL1A1*, *COL3A1* and *FN1* at 48 hours (Figure 1). TGF-β1 increased α-SMA protein levels at 48 and 72 hrs (Figure 2A) and those of COL1A1 only at 72 hrs (Figure 2). In addition, protein levels of COL3A1 increased significantly at 24, 48 and 72 hours while those of FN1 increased at 12, 48 and 72 hrs (Figure 2). These data establish the timeframe for optimum differentiation of myofibroblasts and consequent ECM production in response to TGFβ1; these experimental conditions are used for subsequent experiments to evaluate the contribution of HuR.

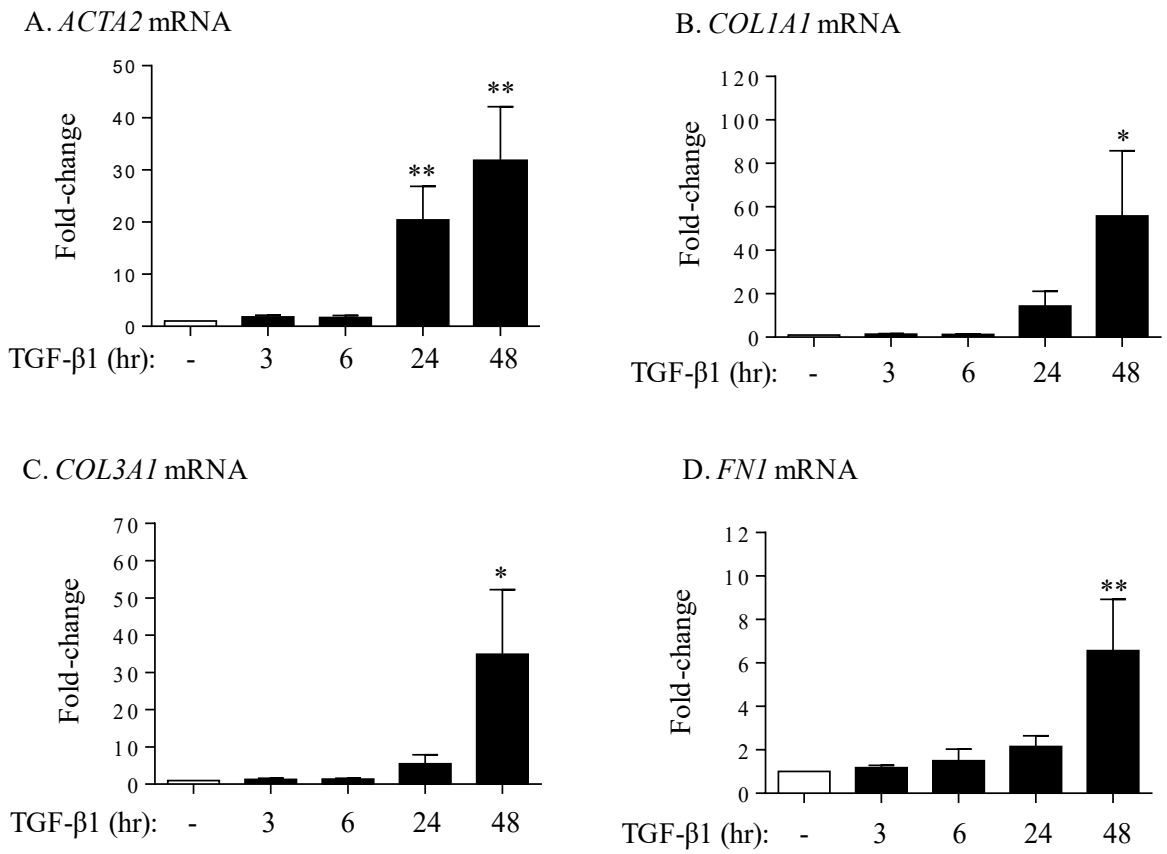

Figure 4.1: Upregulation of mRNA levels of *ACTA2*, *COL1A1*, *COL3A1* and *FN1* by TGFβ1. TGFβ1 (5μg/ml) increases mRNA levels of *ACTA2* (A), *COL1A1* (B), *COL3A1* (C) and *FN1* (D) in HLFs in a time-dependent fashion. Values are means ± SEM (n = 6 per time point). *p< 0.05 and **p< 0.01, as compared to control.

Figure 2

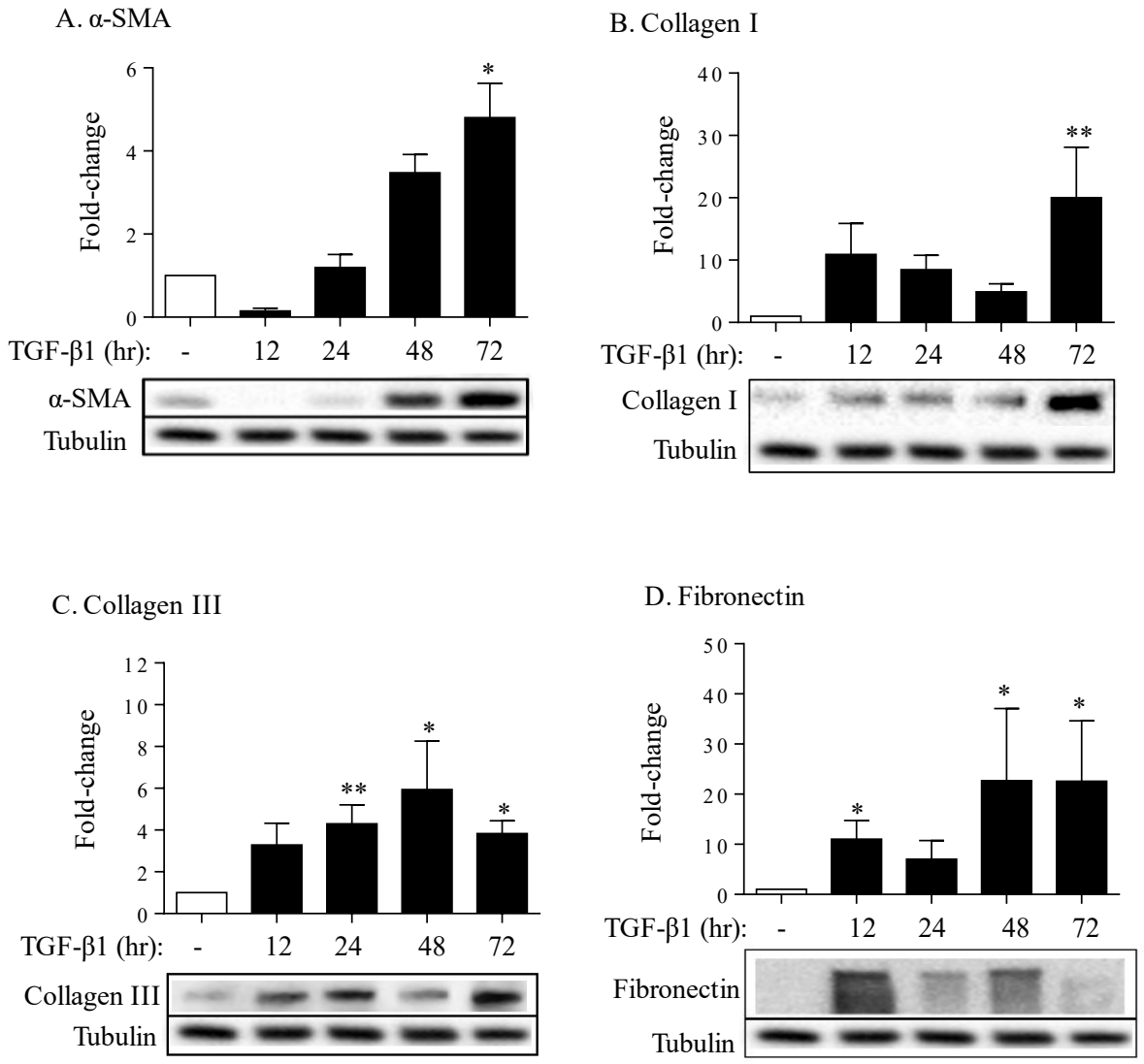

Figure 4.2: Upregulation of protein levels of α-SMA, Collagens and Fibronectin by TGFβ1 in HLFs. TGFβ1 (5μg/ml) significantly increases protein levels of α-SMA (A), Collagen I (B), Collagen III (C) and Fibronectin (D) in HLFs in a time-dependent fashion. Values are means ± SEM (n = 6 per time point). *p< 0.05 and **p< 0.01, as compared to control.

4.2 TGFβ1 increases HuR translocation to the cytosol: The effect of TGFβ1 on HuR expression and intracellular localization is unknown. Hence, we treated HLFs derived from normal subjects with TGFβ1 (5μg/ml) from 12-72 hrs and evaluated HuR mRNA (*ELAVL1*) and protein levels as well as cellular localization. TGFβ1 did not alter total mRNA or protein expression of HuR (Figure 3).

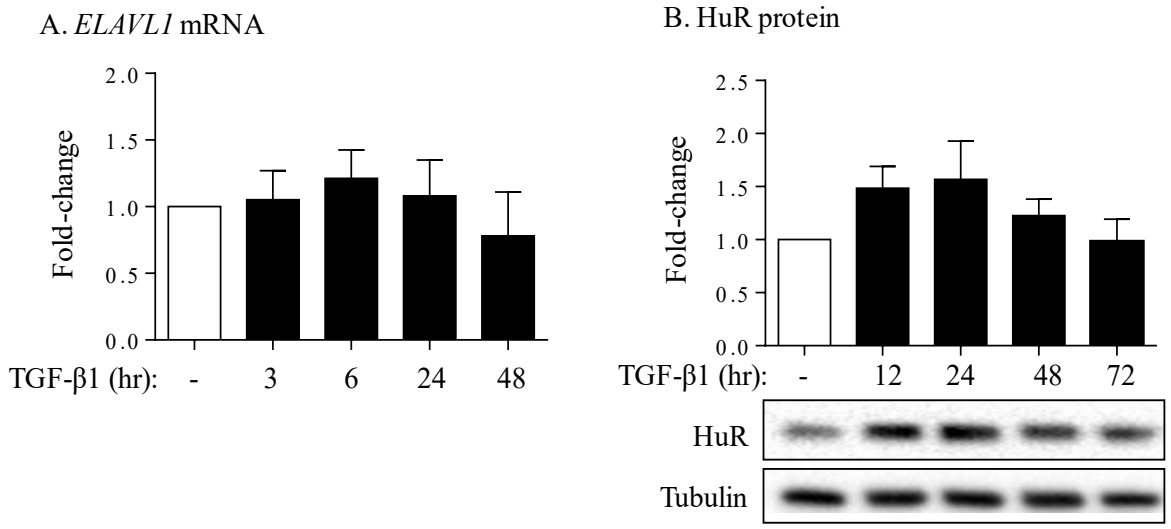

Figure 4.3: HuR expression remains unchanged in response to TGFβ1 in HLFs. mRNA (A) and protein (B) levels of HuR was assessed in HLFs exposed to TGFβ1 for different time points. Note that TGF-β1 exposure did not alter HuR mRNA (*ELAVL1*) or protein expression. Values are means ± SEM (n = 6 per time point).

In order to stabilize target mRNA, HuR must translocate from the nucleus to the cytoplasm [94, 95]. Therefore, we next assessed cytoplasmic translocation of HuR using two experimental approaches. First, we treated HLFs with TGFβ1 for 6 or 24 hrs and then separated total cell lysates into cytosolic and nuclear fractions. Both fractions then underwent western blotting to assess HuR protein levels. There was an increase in cytosolic levels of HuR by about six and two-fold after 6 or 24 hrs exposure to TGFβ1, respectively (Figure 4A). Second, we assessed nuclear versus cytoplasmic localization of HuR using IF. Figure 4B

illustrates that there is a noticeable increase in cytosolic HuR after TGFβ1 exposure. ActD, used as a positive control, elicited dramatic increase in cytosolic HuR localization (Figure 4B- *right panel*). Quantification of cytoplasmic levels revealed that there was a significant increase in cytoplasmic HuR (Figure 4C). Taken altogether, these results indicate that TGFβ1 induces HuR cytoplasmic translocation without changing total HuR cellular expression.

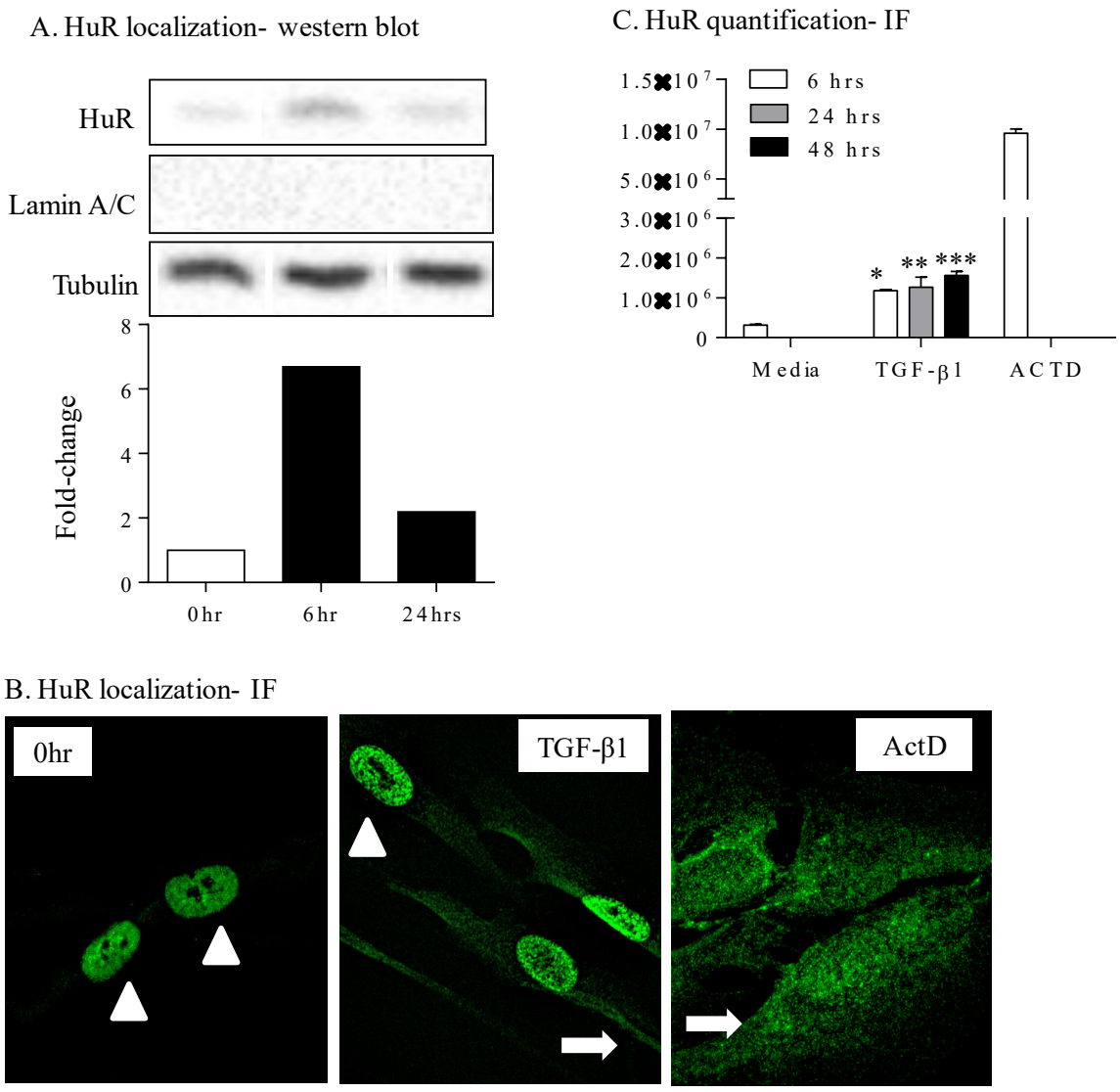

Figure 4.4: **TGFβ1 increases HuR translocation to the cytosol.** (A) HuR localization-western blot: Western blot and quantification of HuR protein in the cytosolic fraction of HLFs treated for 6 or 24 hrs with TGFβ1 (5μg/ml). Note the increase in HuR cytosolic protein levels in response to 6 hrs of TGFβ1 exposure. The absence of Lamin A/C protein (nuclear marker) confirms purity of the cytosolic fraction. Tubulin was used as a loading

control. (B) HuR localization- IF: Nuclear and cytosolic localization of HuR was detected in HLFs without and with TGFβ1 treatment (48 hrs). Arrowheads designate nuclear staining while white arrows indicate cytosolic staining. Note the increase in cytosolic HuR expression in response to TGFβ1 and ActD. Images are representative of two independent experiments. (C) HuR quantification- IF: quantification of HuR expression in the HLFs cytosol-treated for 6, 24 and 48 hrs with TGFβ1 (5μg/ml). Note the increase in HuR cytosolic expression levels in response to TGFβ1 exposure in a time-dependent manner.

4.3 HuR enhances fibroblast differentiation into myofibroblasts: α-SMA and ECM markers are elevated in fibrotic lung disease, an effect which has been attributed to the increase in TGFβ1 levels. It is possible that the effect of TGFβ1 on fibroblast differentiation into myofibroblasts is mediated through binding of HuR α-SMA and ECM mRNA, resulting in stabilization of these transcripts. To assess this, we knocked-down HuR in HLFs using siRNA oligos and assessed the effect of TGF-β1 (5μg/ml) on the expression of α-SMA and ECM markers. Scrambled siRNA oligos were used as control (siCtrl). We verified that transfection of HLFs with HuR-specific siRNA oligos triggered more than a 50% decrease in HuR mRNA and protein levels in HLFs untreated and treated for different time points with TGFβ1 (Figure 5A and 5B). Reducing HuR expression resulted in significant attenuation of TGFβ1-induced increase in α-SMA (ACTA2) mRNA and protein levels (Figure 5C and 5D). Unlike these changes in α-SMA, knocking down HuR expression showed no consistent effects on ECM mRNA expression in response to TGFβ1 (Figure 6). These data indicate that knock-down of HuR affects TGFβ1-induced fibroblasts differentiation.

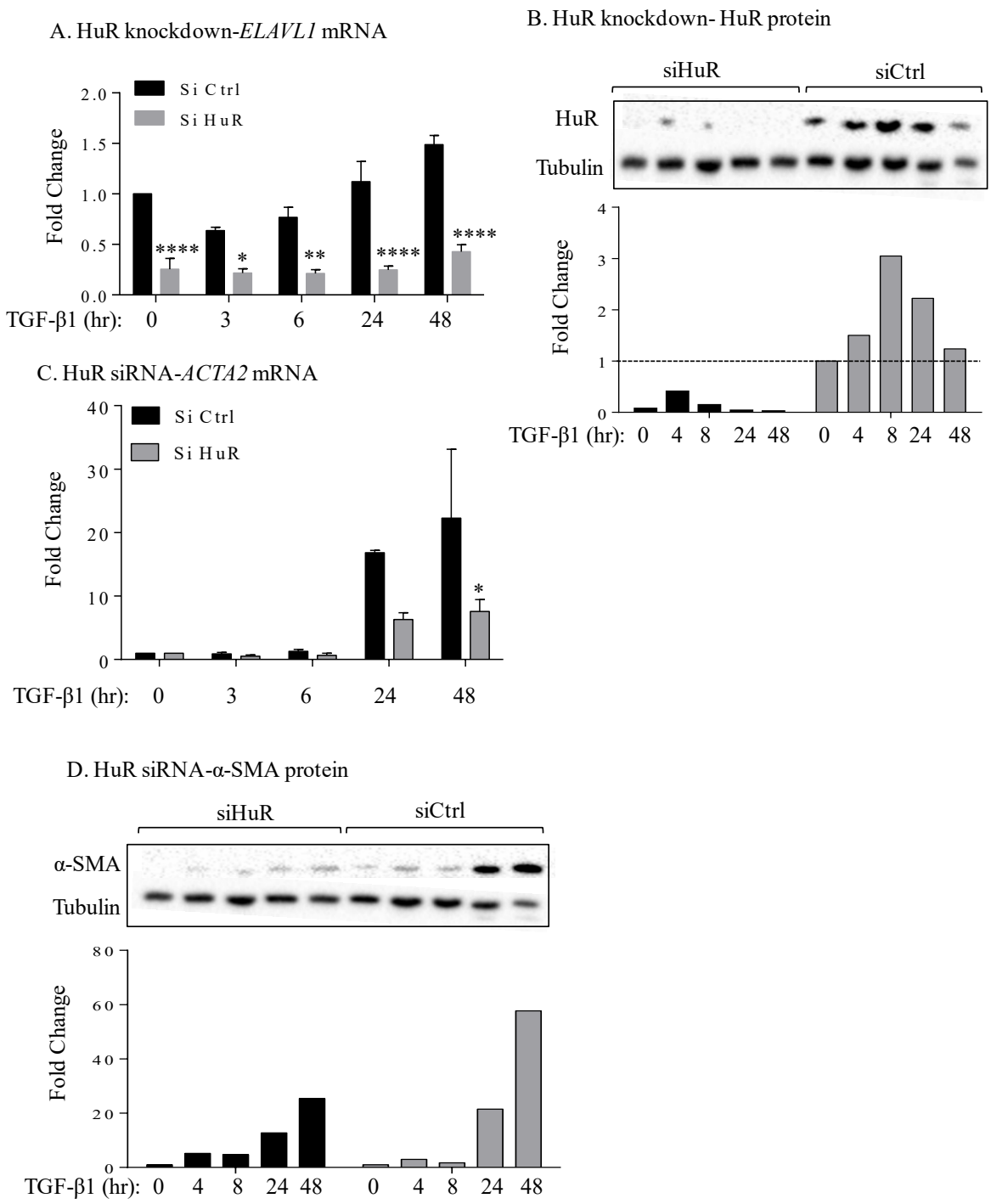

Figure 4.5: HuR knockdown attenuates TGFβ1-induced increase in α-SMA expression in HLFs. *ELAVL1* mRNA (A) and HuR protein (B) levels in HLFs transfected with control siRNA and HuR siRNA oligos and treated with TGFβ1. *ELAVL1* mRNA values (means ± SEM) are expressed as fold change from values measured in cells transfected with control siRNA and untreated with TGFβ1. Note the significant decrease in HuR expression in cells transfected with HuR siRNA oligos (*p< 0.05, **p< 0.01, and ****p<0.001 compared to control siRNA oligos). ACTA2 mRNA (C) and α-SMA (D) protein levels were also evaluated in in HLFs transfected with control siRNA and HuR siRNA oligos. Note the

significant attenuation of TGFβ1-induced increase in α-SMA (*ACTA2*) expression (*p< 0.05, as compared to control siRNA oligos).

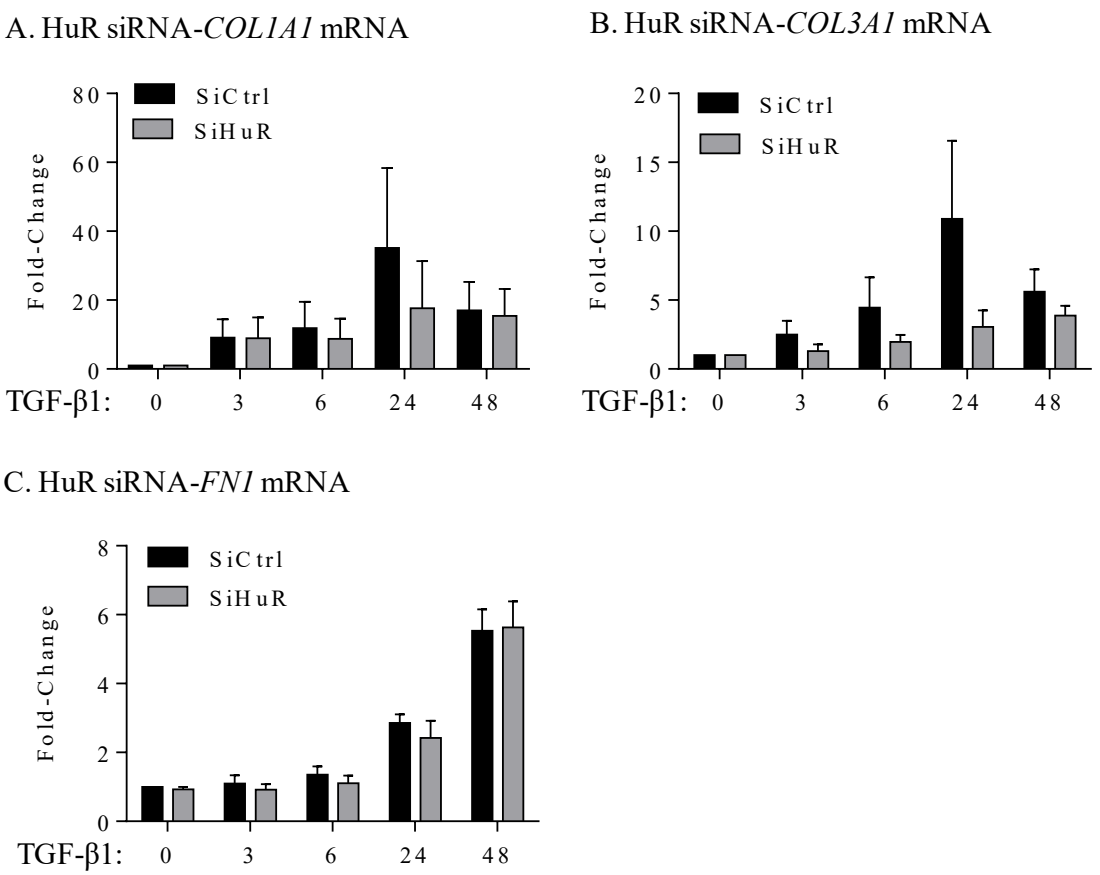

Figure 4.6: HuR knockdown does not affect TGFβ1-induced increase in *COL1A1*, *COL3A1* or *FN1* mRNA expression in HLFs. *COL1A1* (A), *COL3A1* (B) and *FN1* (C) mRNA expression was evaluated in TGFβ1-treated HLFs transfected with control siRNA (SiCtrl) and HuR siRNA (SiHuR) oligos mRNA values (means± SEM) are expressed as fold change from values measured in cells transfected with control siRNA and untreated with TGFβ1.

4.4 HuR knockdown does not affect mRNA stability of ACTA2, COL1A1, COL3A1 or FN1 genes: Our study indicates that HuR knockdown attenuated TGFβ1-mediated increase in α-SMA (*ACTA2*) expression but had no effect on the induction of mRNA for other ECM markers induced by TGFβ1. In the following experiments, we evaluated whether HuR plays a role in stabilizing mRNA of α-SMA or the ECM markers *COL1A1*, *COL3A1* or *FN1* in HLFs exposed to TGFβ1. HLFs transfected 24 hrs earlier with control or HuR siRNA oligos were treated with TGFβ1 (5μg/ml) for 24 hrs after which the cells were treated with ActD (1 μg/ml) to inhibit mRNA transcription; mRNAs were quantified by qPCR 1, 3 and 6 hrs after ActD treatment (Figure 7A). HuR knockdown had no significant effect on mRNA levels of *ELAVL1* (Figure 7B), *ACTA2* (Figure 7C) or the ECM markers *COL1A1* (Figure 7C), *COL3A1* (Figure 7D) or *FN1* (Figure 7E) measured after 1, 3 and 6 hrs of ActD treatment. These data suggest that HuR does not influence mRNA stability of α-SMA (*ACTA2*) or ECM markers in HLFs treated with TGFβ1.

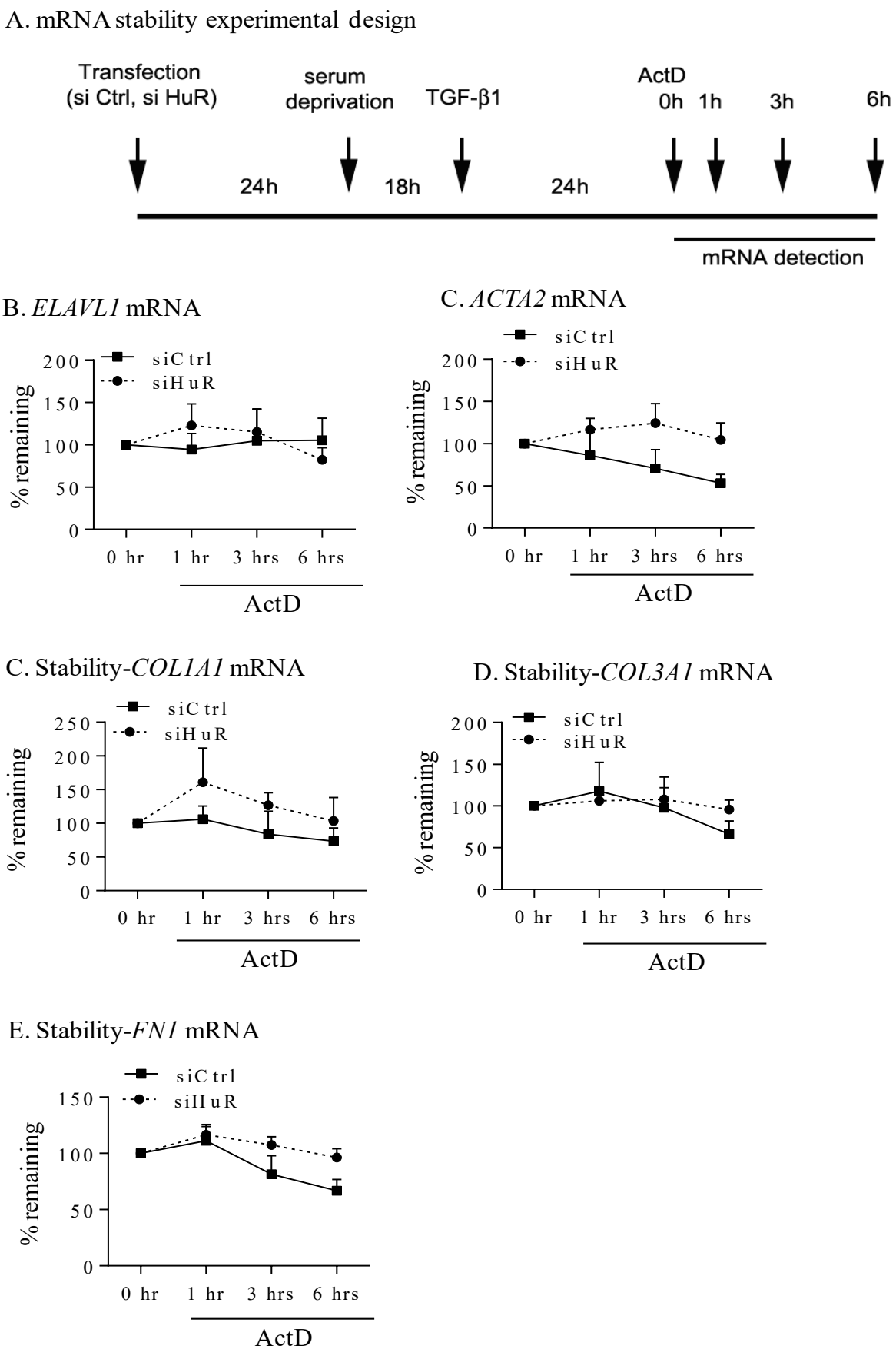

Figure 4.7: HuR does not influence mRNA stability of *ACTA2* or the ECM markers *COL1A1*, *COL3A1* or *FN1*. (A) Experimental protocol for measuring the role of HuR in

mRNA stability. Note that 0 hr refers to the time point immediately after the addition of ActD. TGFβ1 and ActD were added at final concentrations of 5 μg/ml and 1 μg/ml, respectively. HLFs transfected 24 hrs earlier with control or HuR siRNA oligos were treated for 24 hrs with 5 n/ml of TGFβ1. Cells were then treated with AcD and mRNA levels of ELVAL1 (B), *ACTA2 (C),* COL1A1 (D), COL3A1 (E) and FN 1 (F) were measured after 1, 3 and 6 hrs using qPCR. Values are means ± SEM and are expressed as percent of values measured at time 0. There was no significant difference in mRNA stability.

4.5 There is enrichment of ACTA2 and ECM mRNA bound to HuR in response to TGFβ1: Next, HuR-immunoprecipitation (HuR-IP) was carried out to verify if HuR interacts with mRNA of α-SMA (*ACTA2*) and/or ECM markers. HLFs were untreated or treated with TGFβ1 for 24 hrs and the cell lysates underwent IP with control IgG or anti-HuR IgG antibodies. IPs then underwent western blotting (verification of HuR-IP) or total RNA extraction for the detection of gene enrichment using qPCR. Figure 8A shows detection of HuR protein in crude cell lysates (Input) and IPs with only anti-HuR IgG antibody (Figure 8A). We also verified that *β-Actin* mRNA (a known mRNA target of HuR [95]) bound up to 80-fold in HuR-IPs compared to (control) IgG-IP (Figure 8B). *ELAVL1* mRNA was also enriched in the HuR-IP but this was not changed by treatment with TGFβ1 (Figure 8C). In HFLs treated with TGFβ1, there was dramatic enrichment of *ACTA2* (Figure 8D), *COL1A1* (Figure 8E) and *COL3A1* (Figure 8F)- but not *FN1* (Figure 8G)- mRNA in HuR-IPs as compared to IgG-IPs. These results suggest that HuR selectively binds to mRNA of *ACTA2*, *COLIA1* and *COL3A1* in response to TGFβ1.

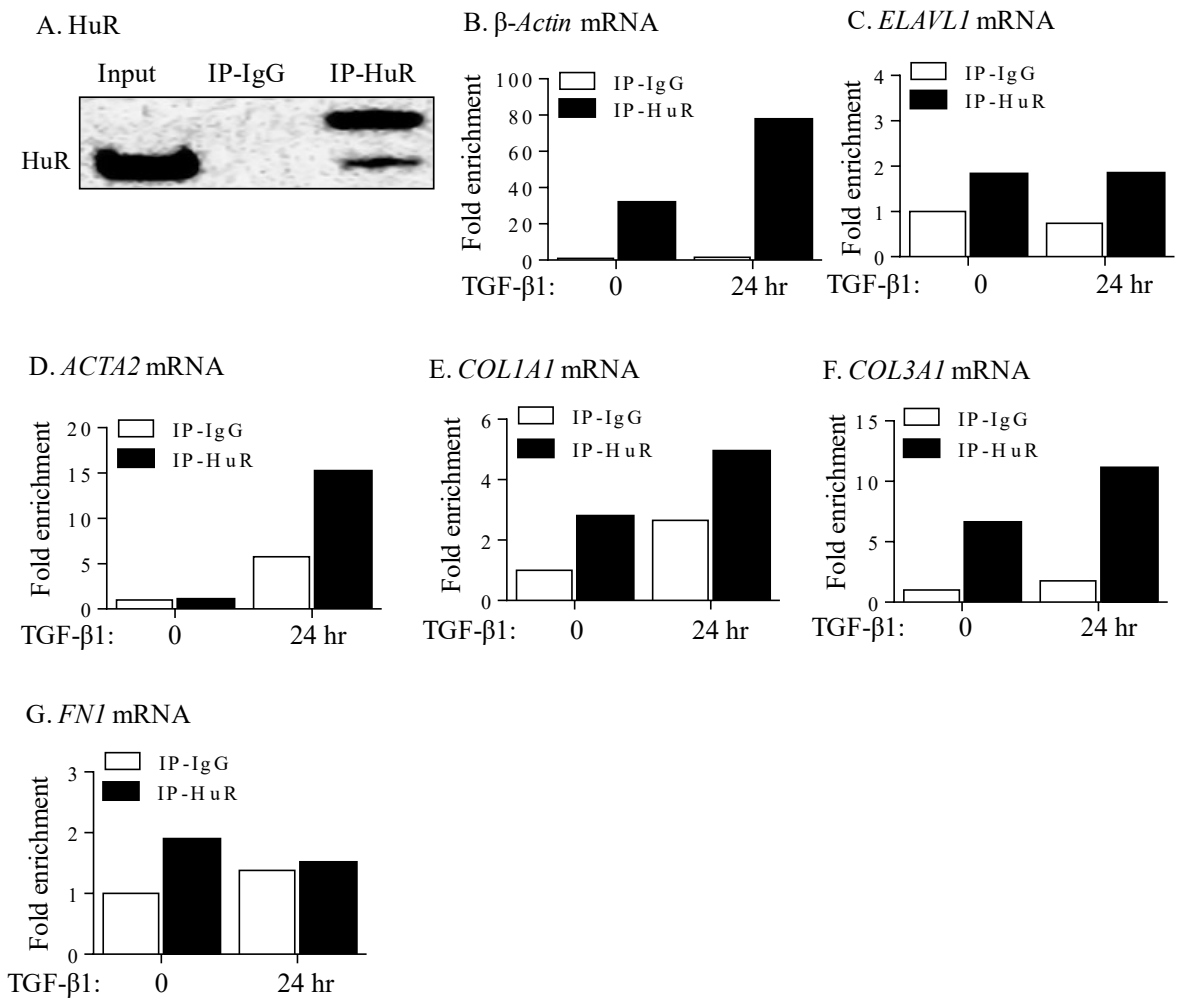

Figure 4.8: Selective binding of HuR to mRNAs of α-SMA and Collagens in HLFs treated with TGFβ1. (A) HuR: Representative western blot of HuR protein levels in HLFs treated with TGFβ1 for 24 hrs. Input refers to crude cell lysates. IP-IgG refers to immunoprecipitation (IP) with control IgG antibody while IP-HuR refers to the IP with anti-HuR IgG antibody. Note the presence of HuR protein in IP-HuR but not in IP-IgG. Detection of mRNA for *β-Actin* (B; positive control), *ELAVL1* (C), *ACTA2* (D), *COL1A1* (E), *COL3A1* (F), and *FN1* (G) in IP-IgG and IP-HUR was done using qPCR. Values are expressed as fold change to values measured in IP-IgG in HLFs untreated with TGFβ1 (0 hr). Note the enrichment of *β-Actin*, *ACTA2*, *COL1A1*, and *COL3A1* only in IP-HuR of cells treated with TGFβ1.

CHAPTER 5: DISCUSSION

Recent studies have highlighted the roles of miRNAs and RBP in a number of diseases and biological processes [96, 97]. We are starting to understand that they are major regulators of the genome and have specific cellular functions. They have been implicated in wide variety of diseases such as cancer, cardiovascular disease, inflammatory diseases and autoimmune diseases. Of the many RNA binding proteins, HuR has received significant attention due to its functional diversity and has already been found to have a significant role in many pathologies, including cancer [81], inflammation [86] as well as cardiac and liver fibrosis [90]. The best-known function of HuR is to regulate mRNA stability, either by promoting the translation of target transcripts or by facilitating their degradation [69]. Although the function of HuR differs according to cell type and cell stimuli [98], it is known to promote cell proliferation, differentiation and angiogenesis.

The earliest reports that describe the role of HuR in cancer cell biology originate from the King and Prescott laboratories. Their studies revealed that HuR expression is significantly upregulated in brain, lung and colon cancers and its expression was linked to increased levels of COX2, VEGF, TGFβ1, IL8 and other cancer-associated proteins [99, 100]. Subsequent studies have revealed that increased cytoplasmic HuR levels were associated with higher tumor grade and poor patient outcomes [68]. HuR was also shown to regulate the expression of several mRNAs encoding pro-inflammatory cytokines in macrophages, endothelial cells and intestinal epithelial cells [80]. Recent studies have focused on studying the roles of HuR in cardiac and liver fibrosis. These studies found a significant increase in HuR levels in Hepatic stellate cells (HSC) and cardiac fibroblasts. This rise in HuR levels correlated with the degree of liver and cardiac fibrosis [59, 90]. Despite this recent progress in elucidating the roles of HuR in cardiac and liver fibrosis, its involvement in lung fibrosis and specifically

idiopathic pulmonary fibrosis (IPF) remain unexplored. In the current study, we established an *in vitro* model of IPF and examined the role of HuR on lung fibroblast differentiation induced by TGFβ1.

IPF is a devastating, progressive, and typically fatal lung disease with a median survival of 3-5 years from diagnosis [101]. Currently, there are no successful pharmacological therapies that improve survival or halt disease progression [14]. IPF is characterized by up-regulation of the pro-fibrotic cytokine TGFβ1 which has been implicated in the recruitment, proliferation and differentiation of fibroblasts and myofibroblast and the enhanced production of ECM proteins including collagens (mainly type I and III), proteoglygans and glycoproteins such as fibronectin [8, 102]. Despite its known roles in many pathologies, the functional importance of HuR in IPF is unclear. Our study is the first to explore the relation between HuR and lung fibroblasts differentiation and ECM deposition (α-SMA, Collagen I (COL1A1), Collagen III (COL3A1) and fibronectin.

We examined the effect of TGFβ1 on the expression of differentiation and ECM markers including α-SMA, COL1A1, COL3A1 and fibronectin in human lung fibroblasts. As reported in the literature, we found that TGFβ1 triggered a significant increase in mRNA and protein levels of α-SMA and ECM markers in a time-dependent manner (Figures 1 and 2). The effect of TGFβ1 on fibroblast differentiation and ECM deposition has been well-established in many physiological processes including wound healing [103] and fibrosis [104]. Indeed, once activated, TGFβ1 induces the differentiation of fibroblasts and promotes the production of ECM [105] via both SMAD-dependent and SMAD-independent pathways [106]. The importance of TGFβ1 in pulmonary fibrosis has been demonstrated by finding increased TGFβ1 levels in bronchoalveolar lavage fluid of IPF patients [107]. Furthermore,

exogenous TGFβ1 can induce pulmonary fibrosis in rat lungs [108], emphasizing the importance of this cytokine in the development of pulmonary fibrosis.

Mobilization of HuR from the nucleus to the cytosol is critical for HuR activity. Cytoplasmic HuR expression levels are known to correlate with poor disease outcome in several cancers, inflammatory conditions and fibrotic diseases [73, 90]. For instance, *Kim et al* showed that despite the absence of a significant direct correlation between total HuR levels and squamous cell lung cancer grade, higher cytoplasmic HuR levels were correlated directly with increased COX2 levels, which were linked to worse prognosis and higher cancer grade and tumor invasiveness [74]. In addition, a study conducted on meningioma cells found that high grade neoplasm was associated with higher cytoplasmic levels of HuR but not nuclear levels [109]. These results indicate that increased cytoplasmic HuR levels have fundamental roles in cellular functions such as stabilizing target mRNAs in cancer cells.

In our human lung fibroblast model, we evaluated the effects of TGFβ1 on total cellular levels and subcellular localization of HuR in human lung fibroblasts. We found that total levels of HuR mRNA and protein (full length molecular mass of 36 kDa) were not affected by TGFβ1 exposure; however, this exposure significantly increased cytoplasmic levels of HuR protein as detected by immunoblotting and immunofluorescence (Figure 4). To our knowledge, we are the first to describe increased HuR cytoplasmic localization by TGFβ1 in lung fibroblasts. Our observations are in agreement with those of *Bai et al*. who reported that TGFβ1 induces increased HuR shuttling to the cytoplasm in cardiac fibroblasts within 6 hours [59]. Using immunoblotting, we also found that the effect of TGFβ1 on cytoplasmic mobilization of HuR was transient and was evident within 6 hours of TGFβ1 addition but only weakly detectable after 24 hours (Figure 4A). By comparison, immunofluorescence measurements indicated that increased cytoplasmic HuR localization by

TGFβ1 was prolonged and still evident at 48 hours of TGFβ1 exposure (Figures 4C & 4B). This contradiction regarding the time course of HuR cytoplasmic mobilization might be due to the higher sensitivity of immunofluorescence as compared to immunoblotting.

HuR is known to be cleaved by Caspase-3 into two products (CPs): HuR-CP1 (24kDa) and HuR-CP2 (8kDa) [110-112] and that the cleaved forms of HuR are fully functional in terms of stabilization of target mRNAs. Indeed, HuR-CP1, by associating with the HuR import factor (transportin 2) TRN2, promotes the cytoplasmic accumulation of HuR and triggers an increase in the half-life of the HuR-mRNA target myogenin that aids in muscle generation [112]. Furthermore, it has been reported in head and neck squamous cell carcinoma that during hypoxic conditions, HuR undergoes caspase-mediated cleavage which enables stabilization of certain mRNAs that have proliferative and differentiative roles in cancer such as *c-myc* [111]. Knowing that TGFβ1 can activate Caspase-3 [113, 114], it is possible that the HuR antibody used for immunofluorescence in our study might have detected the cleaved forms of HuR protein while that used in immunoblotting experiments could have detected the uncleaved form of the HuR protein. This possibility is hard to validate since there are no specific antibodies that are designed to detect only the cleaved forms of HuR protein. Furthermore, although the cleaved form of HuR can be detected by immunoblotting if 30-40 μg of protein extract are used, our current study uses 10 μg of protein extract and thus can only detect the full length HuR, thereby also accounting for the contradiction observed above. Although the exact mechanism through which TGFβ1-induces HuR cytoplasmic translocation is unknown, we hypothesize that it may involve the activation of the p38 MAPK pathway. This hypothesis is based on several reports indicating that TGFβ1 activates several MAPK pathways including p38 [115-117]. In a study conducted on vascular endothelial cells, TGFβ1 activated p38 MAPKs [115]. In primary interstitial lung fibroblasts, TGFβ1 induced cell proliferation through phosphorylation of p38 and JNK (c-Jun

amino-terminal kinases), but not the ERK1/2 (extracellular signal-regulated kinases) pathways [116]. It should also be noted that HuR phosphorylation by p38 MAPK increases HuR cytoplasmic translocation through HuR nucleocytoplasmic shuttling domain (HNS) as shown in colon cancer and human bone osteosarcoma cell line [118].

An important hypothesis for our study is that HuR plays a role in TGFβ1-mediated lung fibrosis and that this role is mediated through the regulation of ECM protein expression and fibroblast differentiation. To evaluate this, we assessed the effect of HuR knockdown using specific siRNAs on TGFβ1-induced fibroblast differentiation. We found that HuR silencing significantly decreased TGFβ1-induced αSMA mRNA and protein levels (Figure 5C and 5D). However, the mRNA levels of ECM markers were not affected by HuR silencing (Figure 6). These findings contradict those of *Bai et al*, who reported that the expressions of *COL1A, COL3A* and *fibronectin* expression decreased significantly following HuR knockdown in cardiac fibroblasts [62]. This discrepancy might be due to differences in cell types (cardiac vs. lung fibroblasts). In addition, although we were able to reduce HuR expression by more than 50% using siRNA oligos, it is possible that the remaining HuR protein is sufficient to maintain the expression of ECM markers at levels similar to those achieved using the control siRNA oligos. Furthermore, we cannot exclude the possibility that TGFβ1 upregulates the expression of ECM markers in lung fibroblasts through non-canonical pathways such as the Ca^{+2}/calmodulin-dependent kinase II (CamK II), which does not require the presence of HuR. This is indeed the case in primary human lung fibroblasts where TGFβ1 stimulates the expression of *COL1A1, COL3A1* and *fibronectin* gene expression through the CamK II pathways but independently of SMAD and MAPK pathways [119].

It is well established that HuR regulates the mRNA stability of its target genes by protecting them from the degradation machinery. We assessed whether HuR stabilizes the

mRNAs of *α-SMA* and ECM markers in human lung fibroblasts stimulated with TGFβ1. To this end, we measured mRNA levels of *α-SMA* and ECM markers in human lung fibroblasts transfected with control or HuR siRNA oligos, and then stimulated the cells with TGFβ1 for 24 hours. After 24 hours, the transcription inhibitor Actinomycin D (ActD) was added and mRNA levels were measured after 1, 3 and 6 hours (Figure 7). We found that silencing HuR expression had no effect on the decay of *α-SMA* and ECM marker mRNAs (Figure 8). This observation suggests that HuR may regulate the expression of α-*SMA* and ECM markers by mechanisms other than increasing their mRNA stability. It is possible that other RNA binding proteins and/or microRNAs may play a role in regulating the stability *α-SMA* and ECM marker mRNAs independently of HuR and/or the TGFβ1 pathway. For instance, in cardiac fibroblasts, miR-33a increases the expression of *COL1A1* and *COL3A1* (*in vivo* and *in vitro*) and that knockdown of miR-33a is sufficient to decrease their expression [120]. This effect of miR-33a on *COL1A1* and *COL3A1* expression is mediated through the p38 MAPK pathway but not through the TGFβ1/SMAD pathway [120] In human dermal fibroblasts, TGFβ1 upregulates the expression of *COL1A1* and fibronectin through activation of the RNA-binding protein PTB (polypyrimidine-tract-binding protein), and knockdown of PTB was associated with a significant decrease in *COL3A1* and *fibronectin* expression [121]. Taken together, our findings support the notion that HuR may play a role in TGFβ1-induced fibroblast differentiation but not ECM production. Future research assessing the roles of other RNA-binding proteins and mRNAs is warranted.

Understanding the functional roles of HuR in regulating human lung fibroblast functions requires the identification of its target mRNAs. Data from different groups indicate that HuR interacts with mRNAs in a cell specific context to either positively or negatively regulate target gene expression [72, 122]. In the MCF7 breast cancer cell line, HuR binds directly to the mRNA of Thrombospondin-1 (*TSP1*) and this binding led to a decrease in

MCT1 (Multiple copies in T-cell lymphoma 1) levels and resulted in a less aggressive tumor [123]. Furthermore, binding of HuR to *Cyclin D1* in mesangial cells treated with Angiotensin II triggers a significant increase in fibrogenic processes inside the kidney and a rise in predisposition to proliferative kidney diseases [124]. To confirm whether HuR directly binds to mRNAs of *α-SMA* and ECM markers in response to TGFβ1 exposure in human lung fibroblasts, we used the technique of RNA- immunoprecipitation assay (RIP) which revealed that HuR interacts directly with *α-SMA* and *COL1A1* and *COL3A1* particularly in cells treated with TGFβ1 (Figure 9). This was not the case for *fibronectin*. This observation is in accordance with previous studies documenting direct binding of HuR to *α-SMA* mRNA in hepatic stellate cells stimulated with TGFβ1 [90]. Interestingly, HuR did not bind to *COL1A1* mRNA in these cells confirming the cell-specific nature of HuR binding to various mRNAs [90]. To our knowledge, our results represent the first report documenting the direct binding of HuR to α-SMA and ECM markers in the context of human lung fibroblasts treated with TGF-β1 and suggest that *α-SMA* and *COL1A1* and *COL3A1* may represent potential novel targets for HuR in IPF.

We should emphasize that although our work is the first to assess the functional importance of HuR in the biological responses of the pro-fibrotic cytokine TGFβ1 in human lung fibroblasts, our study has several limitations. First, we used cells isolated from lung tissue of a single subject, which did not allow us to explore the variability of HuR expression and function among different human subjects. Second, we only assessed the functional roles of HuR *in vitro* in response to a single pro-fibrotic stimulus, TGFβ1. It would be interesting to test other known pro-fibrotic stimuli such as radiation to evaluate the role of HuR on fibrogenesis using *in vitro* fibrotic models. It would have been very informative if the roles of HuR were assessed in various *in-vivo* models of fibrosis such as those using TGFβ1-over expression, radiation or bleomycin treatment. The role of HuR in these models could be

assessed using *Elavl 1*$^{fl/fl}$ mice, which provide an interesting model to achieve *in vivo* cell-specific deletion of HuR. Conditional *HuR* knockouts could be achieved using tamoxifen administration of *Elavl 1*$^{fl/fl}$ *Col1α2Cre* mice which would produce a fibroblast specific HuR knockout or via intranasal administration of adenovirus-mediated Cre recombinase to achieve a lung/airway specific knockout. Despite these limitations, our study provides an important framework for future studies designed to improve our understanding of the role of HuR in the fibrotic processes.

In summary, we report for the first time how TGFβ1 affects cellular levels and subcellular localization of HuR in human lung fibroblasts. We found that TGFβ1 has no effect on total HuR levels in these cells, but it significantly increases cytoplasmic translocation of HuR. We also demonstrate that HuR is required for TGFβ1-induced myofibroblasts differentiation with little effect on ECM markers. Our findings support the notion that HuR may be responsible for promoting the development of lung fibrosis and that targeting HuR may prove to be beneficial in preventing the progression of fibrotic diseases such as IPF.

LIST OF ABBREVIATION

Abbreviation	*Meaning*
AEC	Alveolar epithelial cell
α-SMA	Alpha smooth muscle actin
AUF1	AU-binding factor 1
ARE	Adenosine -and- uridine rich elements
AMPK	AMP-activated protein kinase
BCL2	B-cell lymphoma-2
BRF1	Butyrate response factor-1
CARM1	Coactivator-associated arginine methyltransferase 1
Cdk1	Cyclin-dependent kinase 1
Chk2	Checkpoint kinase 2
COX2	Cyclooxygenase-2
Co-SMAD	Common-SMAD
CTGF	Connective tissue growth factor
ECM	Extracellular matrix
EMT	Epithelial- mesenchymal transition
ELAVL1	Embryonic lethal abnormal vision like- 1
GERD	Gastroesophageal reflux
GM-CSF	Granulocyte macrophage-colony stimulating factor
HIF1α	Hypoxia inducible factor 1 alpha
HNS	HuR- nucleocytoplasmic shuttling
HuR	Human Antigen R
HnRNPD	Heterogeneous nuclear ribonucleoprotein D
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
IIP	Idiopathic interstitial pneumonia
IL	Interleukin
IGFBP5	Insulin-like growth-factor-binding protein 5
IBD	Inflammatory bowel disease
IRES	Internal ribosomal entry side
iNOS	Inducible nitric oxide synthase
IFNγ	Interferon gamma
KSRP	KH-type splicing regulatory protein
LAP	Latency associated peptide
miRNA	Micro-RNA
MCL1	Induced myeloid leukemia cell differentiation protein
MMP9	Matrix metalloproteinases 9
MUC5B	Mucin 5B promoter region
MAPK	Mitogen-activated protein kinase
ncRNA	Non-coding RNA
NSIP	Non-specific interstitial pneumonia
PDGF	Platelet derived growth factor
PDGFR	PDGF-receptor
PKC	Protein kinase C
R-SMAD	Receptor-regulated-SMAD

RBP	RNA-binding protein
RRM	RNA-recognition motif
RISC	RNA-induced silencing complex
SPC	Surfactant protein C
SMAD	Homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the *Caenorhabditis elegans* protein Sma
TERT	Telomerase reverse transcriptase
TERC	Telomerase RNA
TGFβ1	Transforming growth factor beta 1
TNFα	Tumor necrotic factor α
TIMP	Tissue inhibitors of matrix metalloproteinases
TβRII	TGFβ-receptorII
TβRI	TGFβ-receptor I
TTR-RBP	Turnover and translation of regulatory RNA-binding proteins
TIA	T-cell intracytoplasmic antigen
TTP	Tristetraprolin
UIP	Usual interstitial pneumonia
UTR	Untranslated region
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

REFERENCES:

.1 Antoniou, K.M., et al., *Interstitial lung disease.* Eur Respir Rev, 2014. **23**(131): p. 40-54.

.2 Demedts, M., et al., *Interstitial lung diseases: an epidemiological overview.* Eur Respir J Suppl, 2001. **32**: p. 2s-16s.

.3 Demedts, M. and U. Costabel, *ATS/ERS international multidisciplinary consensus classification of the idiopathic interstitial pneumonias.* Eur Respir J, 2002. **19**(5): p. 794-6.

.4 Raghu, G., F. Nyberg, and G. Morgan, *The epidemiology of interstitial lung disease and its association with lung cancer.* Br J Cancer, 2004. **91 Suppl 2**: p. S3-10.

.5 Bouros, D. and A. Tzouvelekis, *Idiopathic pulmonary fibrosis: on the move.* Lancet Respir Med, 2014. **2**(1): p. 17-9.

.6 Lynch, J.P., 3rd, et al., *Idiopathic Pulmonary Fibrosis: Epidemiology, Clinical Features, Prognosis, and Management.* Semin Respir Crit Care Med, 2016. **37**(3): p. 331-57.

.7 Martin, M.D., J.H. Chung, and J.P. Kanne, *Idiopathic Pulmonary Fibrosis.* J Thorac Imaging, 2016. **31**(3): p. 127-39.

.8 Spagnolo, P., et al., *Idiopathic pulmonary fibrosis: an update.* Ann Med, 2015. **47**(1): p. 15-27.

.9 Ley, B. and H.R. Collard, *Epidemiology of idiopathic pulmonary fibrosis.* Clin Epidemiol, 2013. **5**: p. 483-92.

.10 Hopkins, R.B., et al., *Epidemiology and survival of idiopathic pulmonary fibrosis from national data in Canada.* Eur Respir J, 2016. **48**(1): p. 187-95.

.11 Gnanapandithan, K., et al., *Gastroesophageal reflux and idiopathic pulmonary fibrosis: A long term relationship.* Respir Med Case Rep, 2016. **17**: p. 40-3.

.12 Naik, P.K. and B.B. Moore, *Viral infection and aging as cofactors for the development of pulmonary fibrosis.* Expert Rev Respir Med, 2010. **4**(6): p. 759-71.

.13 Hoyne, G.F., et al., *Idiopathic pulmonary fibrosis and a role for autoimmunity.* Immunol Cell Biol, 2017. **95**(7): p. 577-583.

.14 Ryu ,J.H., et al., *Idiopathic pulmonary fibrosis: evolving concepts.* Mayo Clin Proc, 2014. **89**(8): p. 1130-42.

.15 Tzilas, V., et al., *Diagnosis of Idiopathic Pulmonary Fibrosis "Pragmatic Challenges in Clinical Practice".* Front Med (Lausanne), 2017. **4**: p. 151.

.16 Oldham, J.M. and I. Noth, *Idiopathic pulmonary fibrosis: early detection and referral.* Respir Med, 2014. **108**(6): p. 819-29.

.17 Cavazza, A., et al., *The role of histology in idiopathic pulmonary fibrosis: an update.* Respir Med, 2010. **104 Suppl 1**: p .S11-22.

.18 Cottin, V., *Interstitial lung disease.* Eur Respir Rev, 2013. **22**(127): p. 26-32.

.19 Raghu, G., et al., *An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline: Treatment of Idiopathic Pulmonary Fibrosis. An Update of the 2011 Clinical Practice Guideline.* Am J Respir Crit Care Med, 2015. **192**(2): p. e3-19.

.20 Raghu, G., et al., *An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management.* Am J Respir Crit Care Med, 2011. **183** :(6)p. 788-824.

.21 Wilson, M.S. and T.A. Wynn, *Pulmonary fibrosis: pathogenesis, etiology and regulation.* Mucosal Immunol, 2009. **2**(2): p. 103-21.

.22 Mathai, S.K., et al., *Incorporating genetics into the identification and treatment of Idiopathic Pulmonary Fibrosis.* BMC Med, 2015. **13**: p. 191.

.23 Kaur, A., S.K. Mathai, and D.A. Schwartz, *Genetics in Idiopathic Pulmonary Fibrosis Pathogenesis, Prognosis, and Treatment.* Front Med (Lausanne), 2017. **4**: p. 154.

.24 Loomis-King, H., K.R. Flaherty, and B.B. Moore ,*Pathogenesis, current treatments and future directions for idiopathic pulmonary fibrosis.* Curr Opin Pharmacol, 2013. **13**(3): p. 377-85.

.25 Todd, N.W., I.G. Luzina, and S.P. Atamas, *Molecular and cellular mechanisms of pulmonary fibrosis.* Fibrogenesis Tissue Repair, 2012. **5**(1): p. 11.

.26 Wolters, P.J., H.R. Collard, and K.D. Jones, *Pathogenesis of idiopathic pulmonary fibrosis.* Annu Rev Pathol, 2014. **9**: p. 157-79.

.27 Selman, M., et al., *Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy.* Ann Intern Med, 2001. **134**(2): p. 136-51.

.28 Barratt, S. and A. Millar, *Vascular remodelling in the pathogenesis of idiopathic pulmonary fibrosis.* QJM, 2014. **107**(7): p. 515-9.

.29 Kendall, R.T. and C.A. Feghali-Bostwick, *Fibroblasts in fibrosis: novel roles and mediators.* Front Pharmacol, 2014. **5**: p. 123.

.30 Barkauskas, C.E. and P.W. Noble, *Cellular mechanisms of tissue fibrosis. 7. New insights into the cellular mechanisms of pulmonary fibrosis.* Am J Physiol Cell Physiol, 2014. **306**(11): p. C987-96.

.31 Thannickal, V.J. and J.C. Horowitz, *Evolving concepts of apoptosis in idiopathic pulmonary fibrosis.* Proc Am Thorac Soc, 2006. **3**(4): p. 350-6.

.32 Li, Y., et al., *Severe lung fibrosis requires an invasive fibroblast phenotype regulated by hyaluronan and CD44.* J Exp Med, 2011. **208**(7): p. 1459-71.

.33 Phan, S.H., *The myofibroblast in pulmonary fibrosis.* Chest, 2002. **122**(6 Suppl): p. 286S-289S.

.34 Moore, M.W. and E.L. Herzog, *Regulation and Relevance of Myofibroblast Responses in Idiopathic Pulmonary Fibrosis.* Curr Pathobiol Rep, 2013. **1**(3): p. 199-208.

.35 Selman, M. and A. Pardo, *Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers.* Proc Am Thorac Soc, 2006. **3** :(4)p. 364-72.

.36 Chapman, H.A., *Epithelial-mesenchymal interactions in pulmonary fibrosis.* Annu Rev Physiol, 2011. **73**: p. 413-35.

.37 Zeisberg, M. and E.G. Neilson, *Biomarkers for epithelial-mesenchymal transitions.* J Clin Invest, 2009. **119**(6): p. 1429.37-

.38 Maharaj, S., C. Shimbori, and M. Kolb, *Fibrocytes in pulmonary fibrosis: a brief synopsis.* Eur Respir Rev, 2013. **22**(130): p. 552-7.

.39 Quan, T.E., S.E. Cowper, and R. Bucala, *The role of circulating fibrocytes in fibrosis.* Curr Rheumatol Rep, 20 :(2)8 .06p. 145-50.

.40 Keeley, E.C., B. Mehrad, and R.M. Strieter, *The role of fibrocytes in fibrotic diseases of the lungs and heart.* Fibrogenesis Tissue Repair, 2011. **4**: p. 2.

.41 Strieter, R.M., et al., *The role of circulating mesenchymal progenitor cells, fibrocytes, in promoting pulmonary fibrosis.* Trans Am Clin Climatol Assoc, 2009. **120**: p. 49-59.

.42 Andersson-Sjoland, A., et al., *Fibrocytes are a potential source of lung fibroblasts in idiopathic pulmonary fibrosis.* Int J Biochem Cell Biol, 2008 :(10)40 .p. 2129-40.

.43 Alhamad, E.H., et al., *Rapid detection of circulating fibrocytes by flowcytometry in idiopathic pulmonary fibrosis.* Ann Thorac Med, 2015. **10**(4): p. 279-83.

.44 Moore, B.B., *Fibrocytes as potential biomarkers in idiopathic pulmonary fibrosis.* Am J Respir Crit Care Med, 2009. **179**(7): p. 524-5.

.45 Biernacka, A., M. Dobaczewski, and N.G. Frangogiannis, *TGF-beta signaling in fibrosis.* Growth Factors, 2011. **29**(5): p. 196-202.

.46 Leask, A. and D.J. Abraham, *TGF-beta signaling and the fibrotic response.* FASEB J, 2004. **18**(7): p. 816-27.

.47 Akhurst, R.J., *Targeting TGF-beta Signaling for Therapeutic Gain.* Cold Spring Harb Perspect Biol, 2017. **9**.(10)

.48 Fernandez, I.E. and O. Eickelberg, *The impact of TGF-beta on lung fibrosis: from targeting to biomarkers.* Proc Am Thorac Soc, 2012. **9**(3): p. 111-6.

.49 Xu, F., et al., *TGF-beta/SMAD Pathway and Its Regulation in Hepatic Fibrosis.* J Histochem Cytochem, 2016. **64**(3): p. 157-67.

.50 Liu, X., H. Hu, and J.Q. Yin, *Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis.* Liver Int, 2006. **26**(1): p. 8-22.

.51 Hata, A. and Y.G. Chen, *TGF-beta Signaling from Receptors to Smads.* Cold Spring Harb Perspect Biol, 2016. **8**.(9)

.52 Krafft, E., et al., *Transforming growth factor beta* 1 *activation, storage, and signaling pathways in idiopathic pulmonary fibrosis in dogs.* J Vet Intern Med, 2014. **28**(6): p. 1666-75.

.53 Khalil, N. and A.H. Greenberg, *The role of TGF-beta in pulmonary fibrosis.* Ciba Found Symp, 1991. **157**: p. 194-207; discussion 207-11.

.54 Allen, J.T. and M.A. Spiteri, *Growth factors in idiopathic pulmonary fibrosis: relative roles.* Respir Res, 2002. **3**: p. 13.

.55 Lasky, J.A. and A.R. Brody, *Interstitial fibrosis and growth factors.* Environ Health Perspect, 2000. **108 Suppl** :4 p. 751-62.

.56 Milosevic, J., et al., *Profibrotic role of miR-154 in pulmonary fibrosis.* Am J Respir Cell Mol Biol, 2012. **47**(6): p. 879-87.

.57 Liu, G., et al., *miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis.* J Exp Med, 2010. **207**(8): p. 1589-97.

.58 Sureshbabu, A., et al., *Relative Roles of TGF-beta and IGFBP-5 in Idiopathic Pulmonary Fibrosis.* Pulm Med, 2011. **2011**: p. 517687.

.59 Bai, D., et al., *A conserved TGFbeta1/HuR feedback circuit regulates the fibrogenic response in fibroblasts.* Cell Signal, 2012. **24**(7): p. 1426-32.

.60 Mukherjee, N., et al., *Integrative regulatory mapping indicates that the RNA-binding protein HuR couples pre-mRNA processing and mRNA stability.* Mol Cell, 2011. **43**(3): p. 327-39.

.61 Gubin, M.M., et al., *Conditional knockout of the RNA-binding protein HuR in CD4(+) T cells reveals a gene dosage effect on cytokine production.* Mol Med, 2014. **20**: p. 93-108.

.62 Abdelmohsen, K. and M. Gorospe, *Posttranscriptional regulation of cancer traits by HuR* .Wiley Interdiscip Rev RNA, 2010. **1**(2): p. 214-29.

.63 Peng, S.S., et al., *RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein.* EMBO J, 1998. **17**(12): p. 3461-70.

.64 Lebedeva, S., et al., *Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR.* Mol Cell, 2011. **43**(3): p. 340-52.

.65 Pullmann, R., Jr. and H. Rabb, *HuR and other turnover- and translation-regulatory RNA-binding proteins: implications for the kidney.* Am J Physiol Renal Physiol, 2014. **30** :(6)6p. F569-76.

.66 Abdelmohsen, K., et al., *Phosphorylation of HuR by Chk2 regulates SIRT1 expression.* Mol Cell, 2007. **25**(4): p. 543-57.

.67 Wang, W., et al., *AMP-activated kinase regulates cytoplasmic HuR.* Mol Cell Biol, 2002. **22**(10): p. 3425-36.

.68 Giaginis, C., et al., *Elevated Hu-Antigen Receptor (HuR) Expression is Associated with Tumor Aggressiveness and Poor Prognosis but not with COX-2 Expression in Invasive Breast Carcinoma Patients.* Pathol Oncol Res, 2017.

.69 Hinman, M.N. and H. Lou, *Diverse molecular functions of Hu proteins.* Cell Mol Life Sci, 2008. **65**(20): p. 3168-81.

.70 Singh, M., et al., *HuR inhibits apoptosis by amplifying Akt signaling through a positive feedback loop.* J Cell Physiol, 2013. **228**(1): p. 182-9.

.71 Katsanou, V., et al ,.*The RNA-binding protein Elavl1/HuR is essential for placental branching morphogenesis and embryonic development.* Mol Cell Biol, 2009. **29**(10): p. 2762-76.

.72 Shi, J.X., et al., *HuR post-transcriptionally regulates TNF-alpha-induced IL-6 expression in human pulmonary microvascular endothelial cells mainly via tristetraprolin.* Respir Physiol Neurobiol, 2012. **181**(2): p. 154-61.

.73 Kotta-Loizou, I., C. Giaginis, and S. Theocharis, *Clinical significance of HuR expression in human malignancy.* Med Oncol, 2014 :(9)31 .p. 161.

.74 Kim, G.Y., S.J. Lim, and Y.W. Kim, *Expression of HuR, COX-2, and survivin in lung cancers; cytoplasmic HuR stabilizes cyclooxygenase-2 in squamous cell carcinomas.* Mod Pathol, 2011. **24**(10): p. 1336-47.

.75 Kim, H.H., et al., *Nuclear HuR accumulation through phosphorylation by Cdk1.* Genes Dev, 2008. **22**(13): p. 1804-15.

.76 Xu, Y.Z., et al., *RNA-binding protein HuR is required for stabilization of SLC11A1 mRNA and SLC11A1 protein expression.* Mol Cell Biol, 2005. **25**(18): p. 8139-49.

.77 Danilin, S., et al., *Role of the RNA-binding protein HuR in human renal cell carcinoma.* Carcinogenesis, 2010. **31**(6): p. 1018-26.

.78 Kakuguchi, W., et al., *HuR knockdown changes the oncogenic potential of oral cancer cells.* Mol Cancer Res, 2010. **8**(4): p.8-520 .

.79 Wang, H., et al., *Preliminary crystallographic analysis of the RNA-binding domain of HuR and its poly(U)-binding properties.* Acta Crystallogr Sect F Struct Biol Cryst Commun, 2011. **67**(Pt 5): p. 546-50.

.80 Srikantan, S. and M. Gorospe, *HuR function in disease.* Front Biosci (Landmark Ed), 2012. **17**: p. 189-205.

.81 Hasegawa, H., et al., *HuR is exported to the cytoplasm in oral cancer cells in a different manner from that of normal cells.* Br J Cancer, 2009. **100**(12): p. 1943-8.

.82 Iruarrizaga-Lejarreta, M., et al., *The RNA-binding protein human antigen R controls global changes in gene expression during Schwann cell development.* J Neurosci, 2012. **32**(14): p. 4944-58.

.83 Abdelmohsen, K., et al., *miR-519 reduces cell proliferation by lowering RNA-binding protein HuR levels.* Proc Natl Acad Sci U S A, 2008. **105**(51): p. 20297-302.

.84 Rivas-Aravena, A., et al., *The Elav-like protein HuR exerts translational control of viral internal ribosome entry sites.* Virology, 2009. **392**(2): p. 178-85.

.85 Jakstaite ,A., et al., *HuR mediated post-transcriptional regulation as a new potential adjuvant therapeutic target in chemotherapy for pancreatic cancer.* World J Gastroenterol, 2015. **21**(46): p. 13004-19.

.86 Katsanou, V., et al., *HuR as a negative posttranscriptional modulator in inflammation.* Mol Cell, 2005. **19**(6): p. 777-89.

.87 Wang, N., et al., *A HuR/TGF-beta1 feedback circuit regulates airway remodeling in airway smooth muscle cells.* Respir Res, 2016. **17**(1): p. 117.

.88 Gregorini, M., et al., *Mesenchymal Stromal Cells Prevent Renal Fibrosis in a Rat Model of Unilateral Ureteral Obstruction by Suppressing the Renin-Angiotensin System via HuR.* PLoS One, 2016. **11**(2): p. e0148542.

.89 Bai, D., et al., *Cytoplasmic translocation of HuR contributes to angiotensin II induced cardiac fibrosis.* Biochem Biophys Res Commun, 2015. **463**(4): p. 1273-7.

.90 Woodhoo, A., et al., *Human antigen R contributes to hepatic stellate cell activation and liver fibrosis.* Hepatology, 2012. **56**(5): p. 1870-82.

.91 Baglole, C.J., et al., *Isolation and phenotypic characterization of lung fibroblasts.* Methods Mol Med, 2005. **117**: p. 115-27.

.92 Zago, M., et al., *Aryl hydrocarbon receptor-dependent retention of nuclear HuR suppresses cigarette smoke-induced cyclooxygenase-2 expression independent of DNA-binding.* PLoS One, 2013. **8**(9): p. e74953.

.93 von Roretz, C., et al., *Apoptotic-induced cleavage shifts HuR from being a promoter of survival to an activator of caspase-mediated apoptosis.* Cell Death Differ, 2013. **20**(1): p. 154-68.

.94 Atasoy, U ,.et al., *ELAV protein HuA (HuR) can redistribute between nucleus and cytoplasm and is upregulated during serum stimulation and T cell activation.* J Cell Sci, 1998. **111 (Pt 21)**: p. 3145-56.

.95 Calaluce, R., et al., *The RNA binding protein HuR differentially regulates unique subsets of mRNAs in estrogen receptor negative and estrogen receptor positive breast cancer.* BMC Cancer, 2010. **10**: p. 126.

.96 Corbett, A.H., *Post-transcriptional regulation of gene expression and human disease.* Curr Opin Cell Biol :52 .2018 ,p. 96-104.

.97 Kaucsar, T., Z. Racz, and P. Hamar, *Post-transcriptional gene-expression regulation by micro RNA (miRNA) network in renal disease.* Adv Drug Deliv Rev, 2010. **62**(14): p. 1390-401.

.98 Cammas, A., et al., *Destabilization of nucleophosmin mRNA by the HuR/KSRP complex is required for muscle fibre formation.* Nat Commun, 2014. **5**: p. 4190.

.99 Dixon, D.A., et al., *Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells.* J Clin Invest :(11)108 .2001 ,p. 1657-65.

.100 Nabors, L.B., et al., *HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3' untranslated regions of cytokine and angiogenic factor mRNAs.* Cancer Res, 2001. **61**(5): p. 2154-61.

.101 Liu, Y.M., K. Nepali, and J.P. Liou, *Idiopathic Pulmonary Fibrosis: Current Status, Recent Progress, and Emerging Targets.* J Med Chem, 2017. **60**(2): p. 527-553.

.102 Bonella, F., S. Stowasser, and L. Wollin, *Idiopathic pulmonary fibrosis: current treatment options and critical appraisal of nintedanib.* Drug Des Devel Ther, 2015. **9**: p. 6407-19.

.103 Liau, G., M.F. Janat, and P.J. Wirth, *Regulation of alpha-smooth muscle actin and other polypeptides in proliferating and density-arrested vascular smooth muscle cells.* J Cell Physiol, 1990. **142**(2): p. 236-46.

.104 Walton, K.L., K.E. Johnson, and C.A. Harrison, *Targeting TGF-beta Mediated SMAD Signaling for the Prevention of Fibrosis.* Front Pharmacol, 2017. **8**: p. 461.

.105 Vaughan, M.B., E.W. Howard, and J.J. Tomasek, *Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast.* Exp Cell Res, 2000. **257**(1): p. 180-9.

.106 Santos, A. and D. Lagares, *Matrix Stiffness: the Conductor of Organ Fibrosis.* Curr Rheumatol Rep, 2018. **20**(1): p. 2.

.107 Molina-Molina, M., et al., *[Quantifying plasma levels of transforming growth factor beta1 in idiopathic pulmonary fibrosis].* Arch Bronconeumol, 2006. **42**(8): p. 380-3.

.108 Sime, P.J., et al ,.*Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung.* J Clin Invest, 1997. **100**(4): p. 768-76.

.109 Gauchotte, G., et al., *Cytoplasmic overexpression of RNA-binding protein HuR is a marker of poor prognosis in meningioma, and HuR knockdown decreases meningioma cell growth and resistance to hypoxia.* J Pathol, 2017. **242**(4): p. 421-434.

.110 Mazroui, R., et al., *Caspase-mediated cleavage of HuR in the cytoplasm contributes to pp32/PHAP-I regulation of apoptosis.* J Cell Biol, 2008. **180**(1): p. 113-27.

.111 Talwar, S., et al., *Caspase-mediated cleavage of RNA-binding protein HuR regulates c-Myc protein expression after hypoxic stress.* J Biol Chem, 2011. **286**(37): p. 32333-43.

.112 Beauchamp ,P., et al., *The cleavage of HuR interferes with its transportin-2-mediated nuclear import and promotes muscle fiber formation.* Cell Death Differ, 2010. **17**(10): p. 1588-99.

.113 Brown, T.L., et al., *Transforming growth factor beta induces caspase 3-independent cleavage of alphaII-spectrin (alpha-fodrin) coincident with apoptosis.* J Biol Chem, 1999. **274**(33): p. 23256-62.

.114 Inman, G.J. and M.J. Allday, *Apoptosis induced by TGF-beta 1 in Burkitt's lymphoma cells is caspase 8 dependent but is death receptor independent.* J Immunol, 2000. **165**(5): p. 2500-10.

.115 Ferrari, G., et al., *TGF-beta1 induces endothelial cell apoptosis by shifting VEGF activation of p38(MAPK) from the prosurvival p38beta to proapoptotic p38alpha.* Mol Cancer Res, 2012. **10**(5): p. 605-1.4

.116 Khalil, N., et al., *Proliferation of pulmonary interstitial fibroblasts is mediated by transforming growth factor-beta1-induced release of extracellular fibroblast growth factor-2 and phosphorylation of p38 MAPK and JNK.* J Biol Chem, 2005. **280** :(52)p. 43000-9.

.117 Tsukada, S., et al., *SMAD and p38 MAPK signaling pathways independently regulate alpha1(I) collagen gene expression in unstimulated and transforming growth factor-beta-stimulated hepatic stellate cells.* J Biol Chem, 2005. **280**(11): p. 10.64-055

.118 Lafarga, V., et al., *p38 Mitogen-activated protein kinase- and HuR-dependent stabilization of p21(Cip1) mRNA mediates the G(1)/S checkpoint.* Mol Cell Biol, 2009. **29**(16): p. 4341-51.

.119 Mukherjee, S., et al., *Ca(2+)/calmodulin-dependent protein kinase IIbeta and IIdelta mediate TGFbeta-induced transduction of fibronectin and collagen in human pulmonary fibroblasts.* Am J Physiol Lung Cell Mol Physiol, 2017. **312**(4): p. L510-L519.

.120 Chen, Z., et al., *MiR-33 promotes myocardial fibrosis by inhibiting MMP16 and stimulating p38 MAPK signaling.* Oncotarget, 2018. **9**(31): p. 22047-22057.

.121 Jiao, H., et al., *TGF-beta1 Induces Polypyrimidine Tract-Binding Protein to Alter Fibroblasts Proliferation and Fibronectin Deposition in Keloid.* Sci Rep, 201 :6 .6p. 38033.

.122 Kim, H.H., et al., *HuR recruits let-7/RISC to repress c-Myc expression.* Genes Dev, 2009. **23**(15): p. 1743-8.

.123 Mazan-Mamczarz, K., et al., *Post-transcriptional gene regulation by HuR promotes a more tumorigenic phenotype.* Oncogene :(47)27 .2008 ,p. 6151-63.

.124 Che, Y., et al., *AngiotensinII induces HuR shuttling by post-transcriptional regulated CyclinD1 in human mesangial cells.* Mol Biol Rep, 2014. **41**(2): p. 1141-50.