THE ROLE OF KRÜPPEL LIKE FACTOR 4 IN IDIOPATHIC PULMONARY FIBROSIS

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John Robert Grimsley
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Committee:

Dr. Geraldine Grant, Thesis Director

Dr. Steve Nathan, Committee Member

Dr. Alan Christensen, Committee Member

Dr. Jim Willett, Director School of Systems Biology

Dr. Timothy Born, Associate Dean for Academic and Student Affairs, College of Science

Dr. Vikas Chandhoke, Dean, College of Science

Date: 7/22/11

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John Robert Grimsley
Bachelors of Science
George Mason University, 2008

Director: Geraldine M. Grant, Associate Professor
Department of Molecular and Microbiology

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DEDICATION

This is dedicated to my wife, Diana, who has kept me motivated and provided unconditional support and love through the duration of graduate school. My thesis is dedicated to my mother, father, brother and sister, Allie, who all have kept me motivated to better the world. It is dedicated to my extended family, all of who have achieved so much with so little serving a inspiration.

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LIST OF ABBREVATIONS/SYMBOLS

AEC ..... …Activated epithelial cell
alpha-SMA ..... alpha-smooth muscle actin
CBP ..... CREB-binding protein
CTGF ..... Connective tissue growth factor
DMEM ..... Dulbecco's Modified Eagle Medium
DOX ..... Doxycycline
ECM ..... Extra cellular matrix
ELISA ..... Enzyme-linked immunosorbent assay
EMT ..... Epithelial-to-mesenchymal transition
FBS ..... Fetal bovine serum
iPS cells ..... induced pluripotent stem cells
ILD ..... Interstitial lung disease
IHC ..... Immunohistochemistry
IFN-gamma ..... Inteferon-gamma
IPF ..... Idiopathic pulmonary fibrosis
KLF ..... Krüpple-like factor
LTRC ..... Lung Research Tissue Consortium
MET ..... Mesenchymal-to-epithelial transition
PCNA ..... Proliferating cell nuclear antigen
PDGF ..... Platelet-derived growth factor
Q-RTPCR ..... Quantitative-real time polymerase chain reaction
SCC ..... Squamous cell carcinoma
TBS ..... Tris-buffered saline
TNF-alpha ..... Tumor necrosis factor-alpha
VSMC ..... Vascular smooth muscle cells

ABSTRACT

THE ROLE OF KRÜPPLE LIKE FACTOR 4 IN IDIOPATHIC PULMONARY FIBROSIS

John Robert Grimsley, MS

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Thesis Director: Dr. Geraldine M. Grant

Idiopathic Pulmonary Fibrosis (IPF) is a fatal interstitial lung disease (ILD) with no known cause and is characterized by a progressive build up fibrotic tissue in the diseased lung. Krüppel-Like Factor 4 (KLF4), a transcription factor with key roles in the cell cycle, cellular differentiation and development, was found to be over-expressed in primary IPF fibroblasts. In this study we sought to investigate the potential role of KLF4 in IPF by investigating the effect of its over-expression on fibroblast differentiation and proliferation status in the normal human pulmonary fibroblasts cell line, MRC5. In addition, we investigated the localization of KLF4 *in vivo* using normal and IPF tissue (LTRC). The *in vivo* localization of KLF4 in IPF and normal tissue was confirmed by double-immunohistochemistry (IHC) in combination with either alpha-SMA or PCNA (Abcam). KLF4 over-expression in MRC5 cells was achieved using a doxycycline-inducible lentiviral system (Addgene) and confirmed by quantitative real time PCR (Q-

RTPCR) and western blot. The effect of KLF4 over-expression on markers of proliferation and activation was monitored by Q-RTPCR. KLF4 in the IPF lung was localized on the perimeter of the fibroblastic foci and in the parenchyma in areas of advanced fibrosis, however, it absent with in the fibrotic foci. Co-localization of KLF4 and PCNA was observed, however, alpha-SMA and KLF4 were not observed to co-localize. Following KLF4 induction *in vitro* a 6-fold increase in KLF4 mRNA and a 3-fold increase in protein were observed. A decrease (2-fold) in alpha-SMA expression at both the gene and protein level was detected. A 3-fold increase in collagen 1A1 (3-fold) mRNA was observed, but of all the selected proliferation markers (PCNA, cyclin D1 and p21 and p53) decreased. The *in vivo* distribution of KLF4, suggests that it may be involved in the advancement of fibrosis and expansion of the foci. *In vitro* over-expression studies in MRC5 cells revealed a profoundly negative effect on the cell cycle, which appears to be in contrast to IPF, however, in keeping with the dual roles of KLF4 as both a tumor suppressor and tumor promoter. Of particular interest was an increase in collagen production, the main ECM component of IPF, as the the result of KLF4 over-expression. KLF4 operated in a strongly context dependent manner, and IPF is a particularly context-driven disease where the ECM acts almost as another pathogenic entity. Therefore, while direct comparison between *in vivo* IPF fibroblasts and *in vitro* MRC5 cells cannot be made, these data indicate a potential role for KLF4 in the pathogenesis of IPF. Further characterization of this role through experimentation in primary adult IPF fibroblasts is warranted.

CHAPTER 1: Introduction

Idiopathic Pulmonary Fibrosis (IPF) is a fatal interstitial lung disease (ILD) with no known etiology and is characterized by a progressive build up fibrotic tissue within the diseased lungs (Dempsey 2006). IPF currently affects over 200,000 Americans between the ages of 55 and 85. There are 50,000 new cases each year and a median life expectancy of 3 to 5 years following diagnosis (National Heart 2010). Currently, there is no cure for IPF and no effective therapy except for transplantation, which is available to very few given the age group. The pathogenic mechanisms of IPF are not well understood, however it is believed to be a disease of dysregulated wound repair. The evolving theory suggests that at some point, following an injury to the alveolar epithelium, an increase in pro-inflammatory and fibro-proliferative mediators invoke a tissue repair response, which becomes unrelenting. In normal lung tissue repair, a highly choreographed and complex event, which involves the recruitment of fibroblasts to the site of injury where they proliferate and differentiate into myofibroblasts (active fibroblasts). Myofibroblasts, characterized by the expression of the cytoskeletal marker, alpha-smooth muscle actin (alpha-SMA) (Scotton 2007), aid in tissue repair by depositing extra cellular matrix (ECM). Once tissue repair is complete in normal lungs, the fibroblasts produce matrix-metalloproteinases (MMPs), which disassemble the ECM, and then the fibroblasts undergo apoptosis (Greenhalgh 1998; Bride 2004). In IPF,

however, fibroblasts continue to accumulate ECM following tissue repair resulting in a build up of fibrotic tissue, thereby distorting the lung architecture and causing the lungs to function improperly and eventually fail (Selman 2001; Noble 2004; Meltzer 2008). IPF is believed to progress through the lung in a wave-like fashion with the leading edge containing the histological hallmark of IPF, the fibrotic foci, which contain numerous myofibroblast (Harari 2010). It is these myofibroblast cells that are believed to be the primary cause of the disease state observed in IPF through their deposition of massive amounts of ECM consisting of predominantly collagen 1, fibronectin, and proteoglycans (Raghu 1985). Additionally, IPF myofibroblasts are believed to be hyper-proliferative and apoptosis-resistant (Selman 2001; Selman 2006; Chambers 2008). Although, the disease mechanism is not understood certain risk factors are believed to be associated with IPF including smoking, infections, environmental pollutants, chronic aspiration and drug exposure (Society 2000).

The origin of the excess of fibroblasts in the IPF lung is not currently defined. However, the IPF lung is thought to be populated by fibroblasts from a number of potential reservoirs. These including resident lung fibroblasts that have become activated (Xia 2008), fibrocytes derived from circulating bone marrow cells (Anderson-Sojland 2008; Moeller 2009), epithelial cells which have transdifferentiated or undergone epithelial-to-mesenchymal transition (EMT) (Marmai; Willis 2007; Kim 2009) and the migration of pleural mesothelial cells into the lung (Nasreen 2009). Currently there is an active debate

in the literature regarding these sources, and it the discussed populations are likely concurrently contributing to the fibroblasts population.

Numerous growth factors and pathways are thought to be dysregulated in IPF and capable of contributing to the diseased state. Amongst the most prominent of those believed to influence the pathogenesis of IPF are, transforming growth factor-beta (TGF-beta) (Khalil 1991; Cao 2000; Khalil 2001; Uhal 2007), platelet-derived growth factor (PDGF) (Cao 2000), connective tissue growth factor (CTGF) (Watts 2004; Tzortzaki 2007), tumor necrosis factor-alpha (TNF-alpha) (Riha 2004; Freeburn 2005), and fibroblast growth factor (FGF) (Khalil 2005). TGF-beta, which is over expressed in both IPF patients (Coker 2001) and animal models (Coker 1997), is perhaps the most significant factor associated with IPF. This secreted and ECM stored protein has been associated with induction of EMT (Kim 2006), induction of myofiroblast differentiation (Carthy 2011), ECM synthesis (Scotton 2007), and induction of CTGF (Sanchez-Elsner 2001). Clearly, TGF-beta is influencing the pathogenesis of IPF and this also implicates the ECM as a potential driver of IPF. However, the exact mechanism, which TGF-beta employs to influences IPF and the degree to which they affect the disease, is not fully understood.

A number of current and revisited theories prevail regarding the pathogenesis of IPF. One such theory suggests that the cause of dysregulated signaling in IPF may reside within the ECM or stems from abnormal alveolar epithelial cell (AEC) signaling (Selman

2001; Phan 2006; Harari 2010). This theory suggests that it is the whole IPF lung milieu, which influences the fibroblasts to function in an aberrant manner. In support of these theories, microarray analysis previously carried out in this lab discovered over 1800 genes that are significantly altered in IPF fibroblasts isolated within 3 hours of surgery compared to normal fibroblasts isolated in the same manner from normal donor lungs (Emblom-Callahan 2010). However, these primary fibroblasts lose much of the gene expression that separates them once placed in culture resulting in gene expression profiles with no significant difference (Emblom-Callahan 2010).

Recently, our lab has identified a 3-fold increase in Krüppel-Like Factor 4 (KLF4) mRNA in IPF fibroblasts relative to normal fibroblasts (Emblom-Callahan 2007). KLF4 is a member of the Krüppel-Like Factor (KLF) family of transcription factors that regulate differentiation, development, proliferation, apoptosis and responses to external stress in various tissues (McConnell 2007; McConnell 2010). The KLF family of transcription factors can bind with co-regulators such as cAMP response element binding protein (CBP) (Song 2002; Evans 2007), Sin3A (Zhang 2001) and p300 (Zhang 2001; Miyamoto 2003) and these complexes can then alter chromatin structure and subsequently, the expression of a wide array of genes. The KLF proteins can also be regulated by post-translational modifications such as phosphorylation (Ouyang 1998; Zhang 2005), ubiquitination (Chen 2005; Banck 2006) and acetylation (Miyamoto 2003; Li 2005; Evans 2007).

KLF4 regulates differentiation, development, proliferation and apoptosis in various tissues and its expression is required for normal skin, colon and eye tissue homeostasis and is essential in epithelial cell differentiation (Segre 1999; Katz 2002; McConnell 2007; Chen 2008; Evans 2008). Originally discovered as a tumor suppressor protein, it is strongly associated with growth arrest (Shields 1996), however, it can also function in a context dependent manner, behaving as both a suppressor and promoter of gene expression.

With respect to the cell cycle, KLF4 is capable of inhibiting expression of cyclin D1 (Shie 2000; Ghaleb 2008), cyclin B (Yoon 2004) and cyclin E (Yoon 2005), all at the transcription level. This regulation typically results in cell cycle arrest at both the G1/S-phase and G2/M-phase checkpoints. Recently, p53 was shown to activate KLF4 following reparable DNA damage, which promoted transactivation of p53 to the p21 promoter, thereby inducing cell cycle arrest (Zhang 2000; Zhou 2009). Furthermore, subsequent to irreparable DNA damage, KLF4 is repressed, which in turn allows p53 to activate the pro-apoptotic target gene *Bax*, resulting in apoptosis (Zhou 2009). These properties classify KLF4 as a tumor suppressor protein, however, it can also act as an proto-oncogene given the right milieu. KLF4's normal inhibition of p53 in cases where p21 is mutated or bypassed is hypothesized to result in unchecked proliferation and cellular protection from apoptosis (Rowland 2005; Rowland 2006). KLF4 over-expression may prevent p53 from activating *Bax* mediated apoptosis and p21 induced cell

cycle arrest if p21 is by-passed or mutated. This mechanism may be at play in IPF fibroblasts, thereby preventing apoptosis and promoting unchecked proliferation.

KLF4 has also been associated in the pathogenesis of numerous cancers where it again acts in a context dependent manner as either a tumor suppressor or an oncogene. In relation to colon (Dang 2003; Zhao 2004), gastric (Wei 2005; Kanai 2006) and lung cancer (Hu 2009; Zhou 2010) loss of KLF4 appears to be a precipitating factor, where it is believed to act as a tumor suppressor. Specifically, in these three cancers KLF4 is down-regulated when compared to normal tissue and increased expression levels correlate with longer survival time and suppression of cell invasion (Dang 2003; Zhao 2004; Wei 2005; Kanai 2006; Hu 2009; Zhou 2010). However, in cutaneous squamous cell carcinoma (SCC) KLF4 is associated with early stages of tumor initiation, progression and metastasis (Huang 2005; Chen 2008). Additionally, in breast cancer KLF4 expression is associated with invasion and migration, and poor prognosis if localized in the nucleus (Huang 2005; Chen 2008; Yu 2011).

Another function of KLF4, and particularly pertinent to IPF, is its regulation of alpha-SMA expression. Studies have reported both increased and decreased expression of alpha-SMA following KLF4 expression, which can occur through a number of different mechanisms, and in a context dependent manner. Specifically, KLF4 can inhibit alpha-SMA expression at the promoter level by direct binding to the TGF-beta control element (TCE) within the promoter (Adam 2000; Liu 2003). It can also inhibit expression via

preventing TGF-beta signaling by binding Smad3 and preventing its binding to the Smad3-binding element (SBE) in the alpha-SMA promoter (Hu 2007). Lastly, KLF4 regulates myocardin, a master regulator of smooth muscle gene expression (Du 2003) through down-regulation and by preventing it from binding to the alpha-SMA promoter (Liu 2005). The ability of KLF4 to regulate alpha-SMA expression is of particular interest to IPF given the role of and high expression levels of alpha-SMA in myofibroblasts. However, emphasizing the context dependent function of KLF4, others report that KLF4 over-expression results in up-regulation of alpha-SMA expression in vascular smooth muscle cells (VSMCs), however, the mechanism by which this occurs in not understood (Wang 2008; Li 2010).

One of the more recently discovered and high profile roles of KLF4 is its involvement, along with two other transcription factors (Sox-2 and Oct-4), in the reprogramming adult differentiated cells to induced pluripotent stem (iPS) cells (Wei 2009). In this process NANOG, a critical factor underlying pluripotency (Pan 2007), must be expressed either through induction or ectopically in order to create iPS cells (Wang 2010). Interestingly, KLF4 over-expression alone can up-regulate NANOG in the absence of Sox-2 or Oct-4, through interaction with the NANOG promoter (Wei 2009; Zhang 2010). Moreover, KLF4 is also essential in the induction of mesenchymal-to-epithelial transition (MET), which is major pathway utilized in the reprogramming of adult terminally differentiated cells to create iPS cells (Li 2010; Wang 2010). This function of KLF4 is of particular interest given the role of EMT in myofibroblasts differentiation in the IPF lung.

In addition to the observed up-regulation of KLF4, IPF fibroblasts also display a 9-fold increase and 3-fold increase in alpha-SMA and p21, respectively, relative to normal fibroblasts (Emblom-Callahan 2007). Given the role of KLF4 in regulating the cell cycle, differentiation and alpha-SMA we set out to investigate the potential role(s) of KLF4 in the pathogenesis of IPF. First, we employed immunohistochenistry (IHC) and tissue from the LTRC, to determine the localization of KLF4 *in vivo*. To further understand KLF4's role in fibroblasts we over-expressed KLF4 in MRC5s, a normal human fibroblast cell line, using a lenti-virus inducible expression system. Subsequently, we analyzed the effect of KLF4 over-expression on the proliferation and activation status of these fibroblasts. These studies revealed a potential role for KLF4 in IPF and further highlight the context dependency KLF4.

CHAPTER 2: Method and Materials

Cell Culture: MRC5 (ATCC CCL-171) cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) plus 10% Fetal Bovine Serum (FBS Valley Biomedical) in 5% CO_2 in a humidified atmosphere. Cells were passed once they had reached approximately 90% confluence. MRC5-hKLF4 cells (described below) were grown in DMEM plus 10% doxycycline reduced FBS (Clontech).

Production of lentiviral vectors: All viral plasmids were obtained from Addgene. Viral component-containing plasmids FUW-M2rtTA (control vector) (Addgene plasmid 20342) (Hockemeyer 2008), FUW-tetO-lox-hKLF4 (gene of interest vector) (Addgene plasmid 20727) (Soldner 2009), pMD2G (envelope plasmid) (Addgene plasmid12259) and psPAX2 (packaging plasmid) (Addgene plasmid 12260) were all expanded in DH5α bacterial cells and isolated by plasmid maxi preparation (Qiagen). Lenti-virus FUW-M2rtTA and FUW-tetO-lox-hKLF4 were produced by transfection in the packaging cell line 293T as follows. Three days prior to transfection, 293T cells were seeded at 1.24 x 10^6 cells per 75cm^2 flask. On day 3 the 293T cells were transfected with either the FUW-M2rtTA plasmid, envelope plasmid (pMD2G) and a packaging plasmid (psPAX2) to generate the control virus, or FUW-tetO-lox-hKLF4 envelope plasmid (pMD2G) and a packaging plasmid (psPAX2) to generate the gene of interest virus. Transfection was carried out using 60μl of Lipofectamine™ as well as the following ratios of each vector to

ensure optimal viral production; 11.25μg for the vector plasmid, 7.3μg for the packaging plasmid, and 3.95μg for the envelope plasmid per T75 flask. The ratios of vectors were determined based on their sequence length. After 4-6 hours of transfection the medium was replaced with fresh DMEM with 10% FBS. To collect packaged FUW-M2rtTA or FUW-tetO-lox-hKLF4 virus the supernatant was collected at 48 and 72 hours post-transfection and stored at 4^{o}C for no longer than 4 days. Virus-containing supernatant was concentrated by employing Vivaspin 20 (Sartorius Stedim) filtration units and centrifugation column at 3,210 x g for ~30 min. This procedure was repeated until the virus was concentrated approximately 20 times. All concentrated virus was stored at -80^{o}C.

Determination of Lentiviral Titer: To determine he titer of the concentrated viral suspension, the p24 capsid protein titer was determined using the Lenti-X™ p24 Rapid Titer Kit (Clontech) per the manufacture's protocol. Additional quantification of the lentiviral titer was carried out via infection of MRC5 cells with either 100μl, 500μl or 1ml of viral concentrate followed by induction of KLF4 expression by addition of doxycycline (DOX). mRNA of DOX induced virally infected MRC5 compared with expression levels in un-infected MRC5s was monitored by quantitative real time reverse transcriptase polymerase chain reaction (Q-RTPCR).

Viral Infection of MRC5 cells: For infection, MRC5 cells were seeded 48 hours prior to infection at a concentration of 4 X 10^5 cells per 60mm^2 cell culture treated dish and allowed to attach overnight at 37^{o}C and 5% CO_2. The following day cells were serum starved for 24 hours to assure synchronization of the cell cycle. For infection 92.5pg of the response virus containing KLF4 and 89pg of the regulatory virus, as determined by p24 ELISA (described above), were diluted to 4ml with DMEM containing 10% reduced DOX serum, added to each dish and then incubated for 24 hours. After 24 hours the media was replaced with fresh DMEM containing 10% reduced DOX serum. These cells were designated MRC5-hKLF4. The new cell line was expanded and frozen down in liquid nitrogen for long-term storage.

KLF4 Induction: MRC5-hKLF4 cells were seeded 48 hours prior to induction at a concentration of 4 X 10^5 cells and 1.25 X 10^6 in 60mm^2 and 100mm^2 dishes respectively. After overnight attachment all cells were synchronized by serum starvation for 24 hours followed by replacing the media with DMEM plus 10% DOX reduced serum containing 2μg/ml DOX to induce KLF4 expression. Negative controls included MRC5-hKLF4 cells without 2μg/ml DOX, and uninfected MRC5 cells in the presence of 2μg/ml DOX. After 24 hours all media was removed and the cells, MRC5-hKLF4 induced and non-induced, as well as MRC5 cells were flash frozen in liquid nitrogen and stored at -80^{o}C for RNA and protein analysis.

Total RNA Extraction: Total RNA was extracted using the RNeasy® Kit (Qiagen) from all induced and non-induced cells, MRC5 and MRC5-hKLF4, after 24 hours of DOX exposure. All RNA was quantified using a Nanodrop™ spectrophotometer (Nanodrop™ 3.0.0, Agilent Technologies) and stored at -80^{o}C.

Quantitative real time polymerase chain reaction (Q-RTPCR): cDNA was generated from 1μg of total RNA using the iScript™ cDNA synthesis kit (Bio-Rad). Q-RTPCR reaction was carried out using SYBR® green PCR kit (Bio-Rad). Gene expression was normalized to ribosomal RNA 18S expression using the Comparative $Ct2^{-(\Delta)(\Delta)Ct}$ method and the fold difference was calculated in comparison to MRC5 cells exposed to DOX for 24 hours and MRC5-hKLF4 cells not exposed to DOX. Gene expression was analyzed for: Krüpple Like Factor 4 (*KLF4*), alpha-Smooth Muscle Actin (alpha-SMA) (*ACTA2*), Cyclin-dependent kinase inhibitor 1 (*p21*), Tumor protein 53 (*TP53*), Proliferation Cell Nuclear Antigen (*PCNA*), Cyclin D1 (*CCND1*), Collagen 1A1 (*COL 1A1*), Fibronectin (*FN1*) and nanog homeobox (*NANOG*.)

18S forward primer: AGGAATTCCCAGTAAGTGCG

18S reverse primer: GCCTCACTAAACCATCCAA

KLF4 forward primer: GCGCTGCTCCCATCTTTCT

KLF4 reverse primer: GGGGGAAGTCGTTCATG

ACTA2 forward primer: GTGTTGCCCCTGAAGAGCAT

ACTA2 reverse primer: GCTGGGACATTGAAAGTCTCA

p21 forward primer: AAATTGGTCAAGGGGCATCAG

p21 reverse primer: ACCCATAGTTCATCACCCACC

TP53 forward primer: CCGCAGTCAGATCCTAGCG

TP53 reverse primer: AATCATCCATTGCTTGGGACG

PCNA forward primer: ACACTAAGGGCCGAAGATAACG

PCNA reverse primer: CGGCATATACGTGCAAATTCAC

CCND1 forward primer: GTGCTGCGAAGTGGAAACC

CCND1 reverse primer: ATCCAGGTGGCGACGATCT

COL 1A1 forward primer: GTCGAGGGCCAAGACGAAG

COL 1A1 reverse primer: CAGATCACGTCATCGCACAAC

FN1 forward primer: AGGAGGACCTCGAAGCAGAG

FN1 reverse primer: GCAGAGTCTGGTTGGCTGTC

NANOG forward primer: TGATTTGTGGGCCTGAAGAAA

NANOG reverse primer: GAGGCATCTCAGCAGAAGACA

Total Protein Extraction: Total cellular protein was isolated using RIPA buffer (Pierce), with addition of complete mini protease inhibitor cocktail tablets (Roche) and PhosSTOP™ phosphatase inhibitors cocktail tablets (Roche) per the manufacture's instructions. Lysed cells were centrifuged at 13,000Xg for 20 minutes to remove cellular debris. The resulting supernatant was collected and stored at -80^{o}C. Total protein concentration was determined by Bradford assay (Bio-Rad).

Western blot analysis: For western blot analysis**,** 20μg of total cellular protein was subjected to gel electrophoresis using a 4-12% Bis-Tris gradient gel (Invitrogen). Visible molecular weight markers were included (Dual Precision Plus Marker, Bio-Rad or EZ-RUN Pre-Stained *Rec* Protein Ladder, Fisher). Proteins were transferred to nitrocellulose membrane using the iBLOT® system (Invitrogen). All membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 1 hour at room temperature. After blocking, the TBS-T was replaced with TBS-T 5% non-fat dried milk containing either alpha-SMA (Abcam, ab7817) or KLF4 (Abcam, ab72543) primary antibody at a concentration of 1μg/ml of each antibody overnight at 4^{o}C. After overnight incubation each membrane was washed 5 times for 5 minutes (5x5mins) with TBS-T, followed by incubation with the appropriate horseradish peroxidase (HRP)-linked secondary antibodies in TBS-T 5% non-fat dried milk for 1 hour at room temperature. Afterward, each membrane was washed with TBS-T 5X5min then visualized by incubation with chemiluminescent Super Signal West Femto Max Sensitivity Substrate (Pierce). For normalization blots were then stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) according to the manufacturer's protocol. Each membrane was then re-probed with beta actin primary antibody (Abcam, ab8227) and secondary antibody as previously described. All immune-blots were imaged using ChemiDoc™ XRS System (Bio-Rad) and densitometry analysis was performed using Quantity One software (Bio-Rad).

Immunohistochemistry: Formalin-fixed, paraffin-embedded IPF and normal tissue slice slides were obtained from the Lung Tissue Research Consortium (LTRC). Serial slides were chosen for multiple analyses. Slides were first deparaffinized and rehydrated in the following series by incubating in 100% xylene for 3minsX2, xylene: 100% ethanol for 3mins (1:1), 100% ethanol for 3minsX2 , 95% ethanol for 3mins, 70% ethanol for 3mins and 50% ethanol for 3mins. Antigen retrieval was performed by pressure-cooking rehydrated slides for 5 minuets in 10mM sodium citrate buffer (pH 6.0). After antigen retrieval, slides were washed twice for 5 minutes in TBS+Triton (0.025%) with gentle agitation. Each slide was then blocked with 1% BSA in TBS for 2 hours at room temperature. Double immunohistochemistry (IHC) was performed by incubation overnight at 4^{o}C in the appropriate primary antibodies diluted in TBS with 1% BSA concurrently (Table 1). Primary antibodies raised in different species were used for double staining. Subsequent to primary incubation, slides were washed 3X5mins in TBS+Triton (0.025%) followed by incubation with species complimentary secondary antibodies conjugated with either Rhodamine or Alexa Fluor® for 1 hour at room temperature in the dark (Table 2). Each slide was then washed 3X5mins in TBS+Triton (0.025%). Nuclei were visualized by counter-staining with DAPI (1μg/ml in PBS) for 1 minute. Slides were then mounted in Cytoseal™ XYL (Richard-Allan Scientific) and covered with a cover slip. Visualization was carried out using a Nikon C1 confocal system mounted on a Nikon Eclipse 90i microscope. Images were processed using Nikon EZ-C1 imaging software and Adobe Photoshop® Elements.

Table 1: Primary antibodies used to perform IHC.

Primary Antibodies	Manufacture/ID
Rabbit polyclonal to KLF4	Abcam/ ab26648
Rabbit polyclonal to KLF4	Abcam/ ab72543
Mouse monoclonal to PCNA	Abcam/ab29
Mouse monoclonal to alpha smooth muscle actin	Abcam/ab7817

Table 2: Secondary antibodies used to perform IHC.

Secondary Antibodies	Manufacture/ ID
Alexa Fluor 488 chicken anti-rabbit IgG	Invitrogen/ A21441
Alexa Fluor 568 Donkey anti-Rabbit IgG	Invitrogen/ A10042
Alexa Fluor 488 goat anti-mouse IgG	Invitrogen/ A21131
Rhodamine (TRITC) 555 goat anti-mouse IgG	Invitrogen/ T-2762

Statistical Analyses:

All statistical analyses were carried out using Prism® 4 software (GraphPad). Paired student t-tests were employed to calculate statistical significance of all Q-RTPCR $2^{\Delta Ct}$ data. Additionally, paired student t-tests were employed to calculate the statistical significance of protein expression data. Mann-Whitney tests were employed to calculate the statistical significance of data generated from primary IPF and normal fibroblasts.

CHAPTER 3: Results

Project rationale and preliminary findings:

The rationale for this project originated from data generated by Dr. Margret Emblom-Callahan [Emblon-Callahan, PhD Dissertation, GMU, 2010], which revealed that non-cultured IPF fibroblasts (n=6) express a 3.2-fold greater level KLF4 mRNA than non-cultured normal fibroblasts (n=4) (Fig. 1) (p-value=.0121).

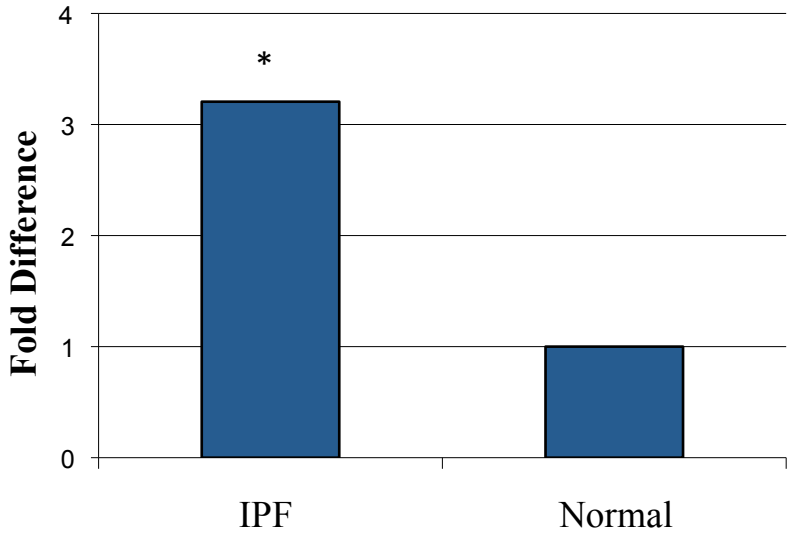

Figure 1: Fold difference of KLF4 mRNA in IPF fibroblasts (n=6) vs. normal fibroblasts (n=4) as determined by Q-RTPCR analysis. * denotes a p value ≤ 0.05 as determined by paired t test.

Viral infection of MRC5 cells:

MRC5 cells were infected with an inducible dual virus system (Hockemeyer 2008; Soldner 2009). Volume per virus correlated with 93pg and 185pg of p24 viral coat protein per 60mm cell culture dish. 24 hours subsequent the infection KLF4 expression was induced for 24 or 48 hours by addition of DOX (2μg/ml). RNA was extracted and Q-RTPCR analysis revealed that induction of cells infected with a viral quantity correlating to 93pg p24 for 24 hour resulted in an 11-fold increase of KLF4 gene expression (Fig. 2). A corresponding 48 hour DOX exposure in these same cells resulted in a 2.5-fold increase KLF4 gene expression (Fig. 2). Induction of cells infected with virus corresponding to 185pg of p24 for 24 hour resulted in a 37-fold increase in KLF4 gene expression, while a 48 hour DOX exposure resulted in a 5-fold increase in KLF4 gene expression (Fig. 2).

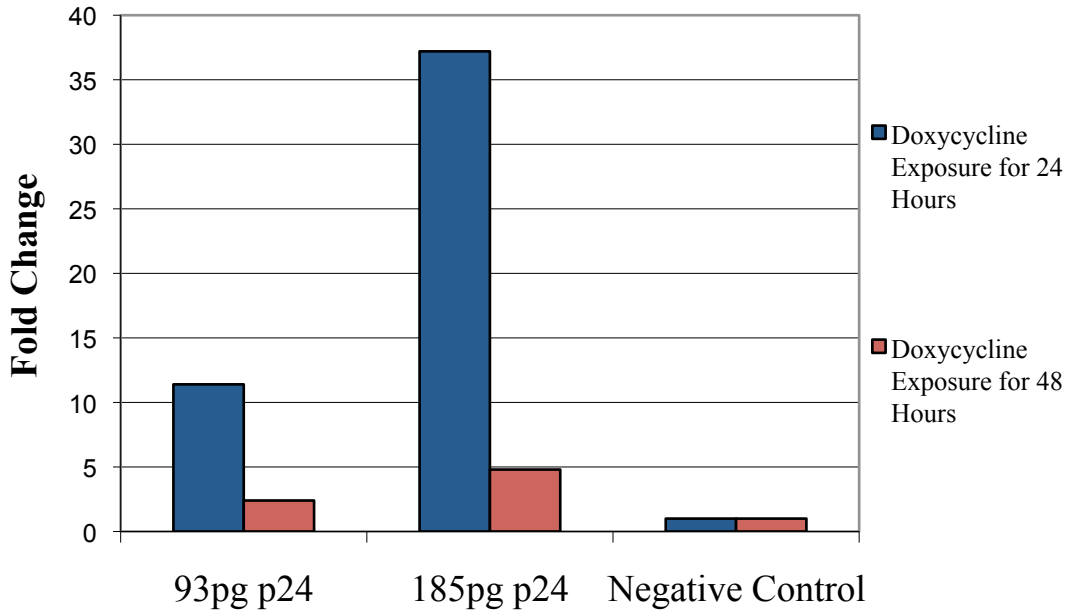

Figure 2: Fold change in KLF4 mRNA following lentivirus infection and DOX exposure in MRC5 cells as determined by Q-RTPCR analysis. MRC5 cells were infected with both the regulator virus (FUW-M2rtTA) and response virus (FUW-tetO-lox-hKLF4) then exposed to DOX for either 24 hours or 48 hours. Uninfected MRC5 cells not exposed to DOX served as the negative control.

KLF4 over-expression:

To remain within the physiological range observed in IPF fibroblasts the viral titer corresponding to 93pg of p24 coat protein and 24 hour DOX exposure was chosen for further experimentation. After exposure mRNA was extracted and KLF4 mRNA levels monitored by Q-RTPCR. The amount of KLF4 expression leakage from the controlled viral system was determined by observing KLF4 gene expression in dual infected MRC5-hKLF4 cells without DOX (-DOX) relative to uninfected MRC5 cells exposed to DOX (+DOX) (2μg/ml). A 1.6 fold increase in KLF4 gene expression was observed in MRC5-hKLF4 -DOX cells relative to MRC5 +DOX cells (Fig. 3) (p-value=.0792).

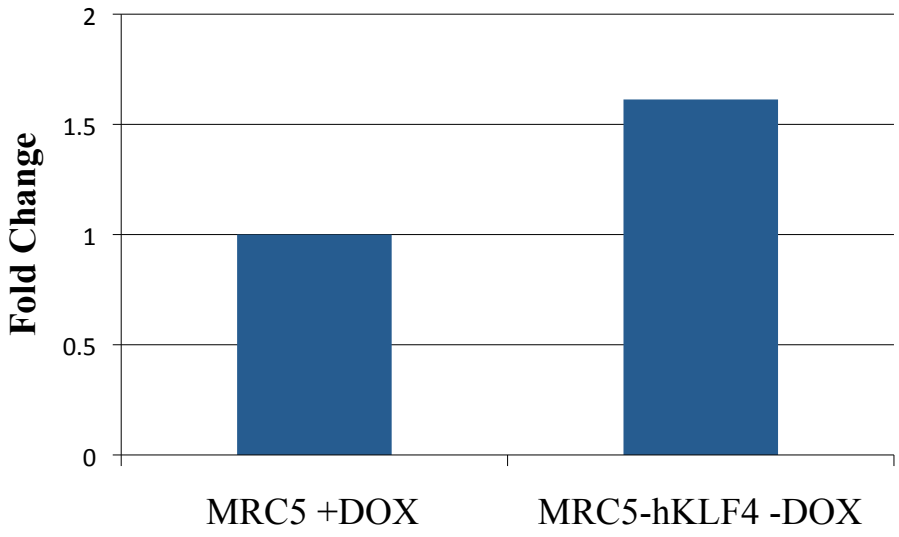

Figure 3: Fold difference of KLF4 expression leakage from MRC5-hKLF4 cells. MRC5-hKLF4 cells in the not exposed to DOX (n=3) compared to parent MRC5 cells exposed to DOX for 24 hours (n=3) as determined by Q-RTPCR analysis.

Once the level of expression leakage of KLF4 had been determined in MRC5-hKLF4 cells the fold change in KLF4 following induction via DOX was determined. A 6.8-fold increase in KLF4 gene expression was observed in MRC5-hKLF4 +DOX cells (n=6) relative to MRC5-hKLF4 -DOX cells (n=3) (Fig. 4) (p-value=.0377). KLF4 protein expression was also examined via western blots at 24 hour post DOX exposure and was found to be 3-fold greater in MRC5-hKLF4 +DOX cells relative to MRC5-hKLF4 -DOX cells (Fig. 5 & 6) (p-value=.0033).

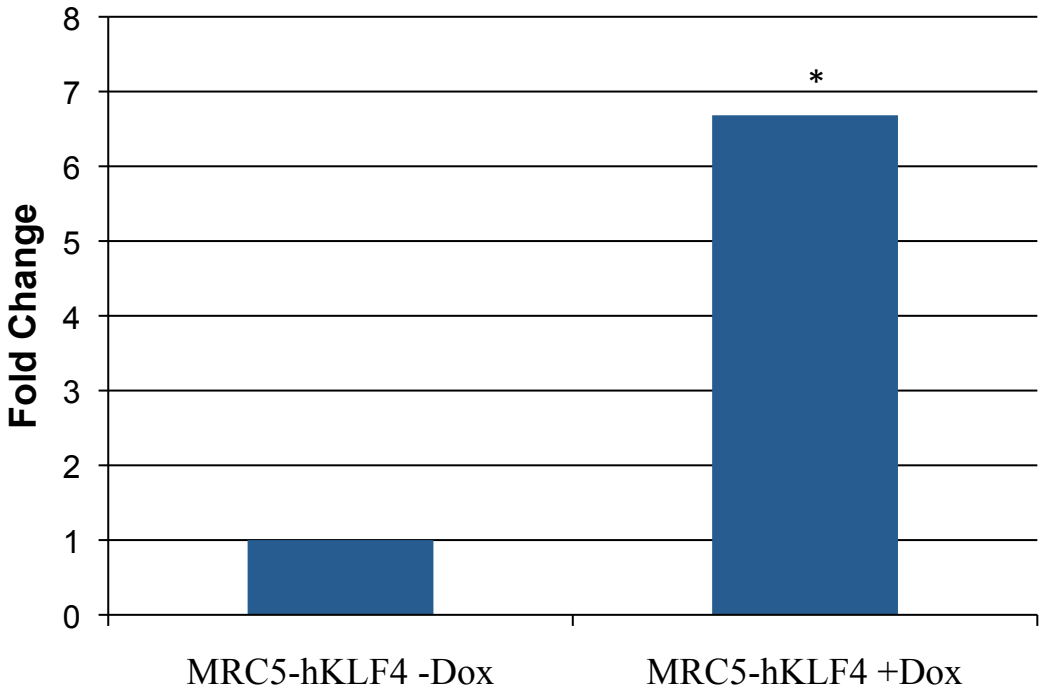

Figure 4: Fold change in KLF4 mRNA in MRC5-hKLF4 cells following DOX exposure for 24 hours (n=6) relative to MRC5-hKLF4 cells with no DOX exposure (n=3) as determined by Q-RTPCR analysis. * denotes a p value ≤ 0.05 as determined by paired t test.

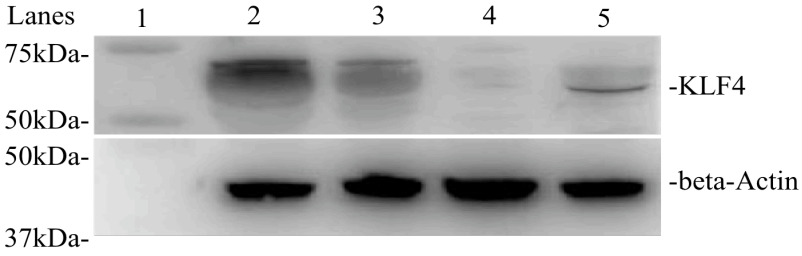

Figure 5: Western Blot of KLF4 protein expression in MRC5-hKLF4 cells following 24 hours DOX exposure. Lane 1: Marker, Lane 2: 20µg whole cell lysate MRC5-hKLF4 cells following DOX exposure for 24 hours, Lane 3: 20µg whole cell lysate MRC5-hKLF4 cells without DOX exposure, Lane 4: 20µg whole cell lysate MRC5 cells exposed to DOX, Lane 5: 20µg whole cell lysate of 293t cells, positive control for KLF4 protein expression. Bottom row is the Beta-actin loading control.

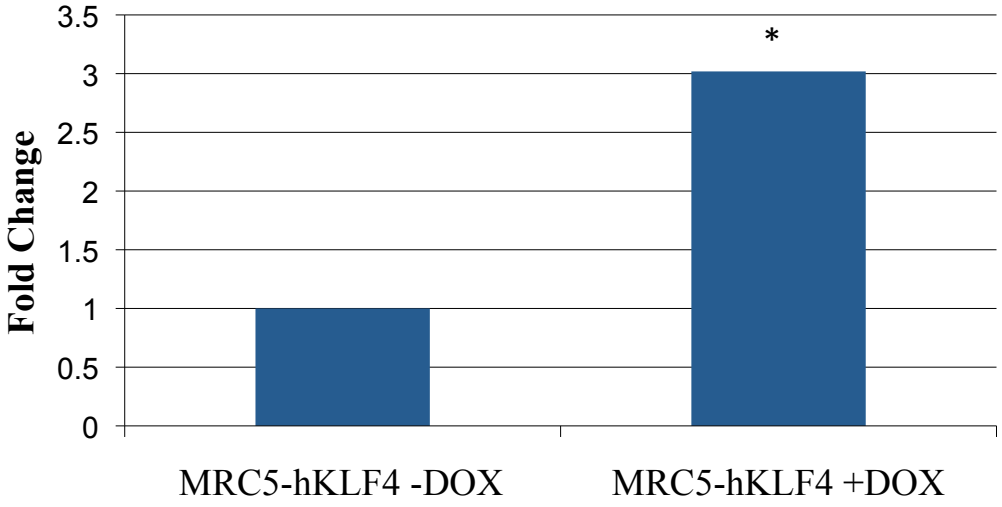

Figure 6: Bar graph of densitometry readings for relative KLF4 protein expression in MRC5-hKLF4 cells following induction for 24 hours. MRC5-hKLF4 cells were exposed to DOX for 24 hours to induce KLF4 over-expression (n=3). Negative control is MRC5-hKLF4 not exposed to DOX (n=3). Samples were normalized to beta-actin protein expression. * p value ≤ 0.05 as determined by paired t test.

Effect of KLF4 over-expression on fibroblast activation markers and ECM production:

To evaluate the affect of KLF4 over-expression on the activation status of MRC5 cells the expression of alpha-SMA, collagen 1A1 and fibronectin were monitored. MRC5-hKLF4 +DOX 24 hr cells displayed a 2.34-fold decrease in alpha-SMA mRNA relative to MRC5-hKLF4 -DOX cells (p-value=.0390) (Fig. 7) and a corresponding 2-fold decrease in protein expression (p-value=.0051) (Fig. 8 & 9). Conversely, collagen 1A1 mRNA increased 3.02-fold and fibronectin mRNA increased 2.14-fold (p-value=.0016) in MRC5-hKLF4 +DOX 24 hr cells (p-value=.0747) (Fig. 7).

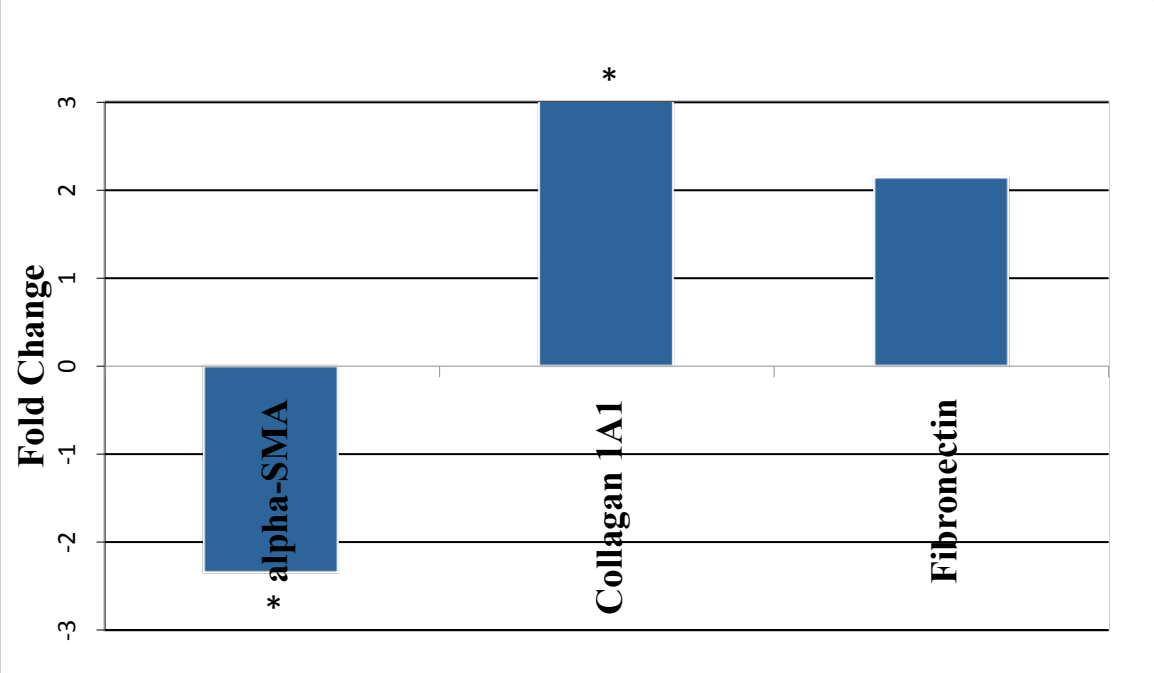

Figure 7: Fold change in mRNA of activation markers (alpha-SMA, Collagen 1A1, Fibronectin) following KLF4 over-expression in MRC5-hKLF4 for 24 hours. MRC5-hKLF4 cells were exposed to DOX for 24 hours to induce KLF4 over-expression. Negative control is MRC5-hKLF4 not exposed to DOX. * denotes a p value ≤ 0.05 as determined by paired t test.

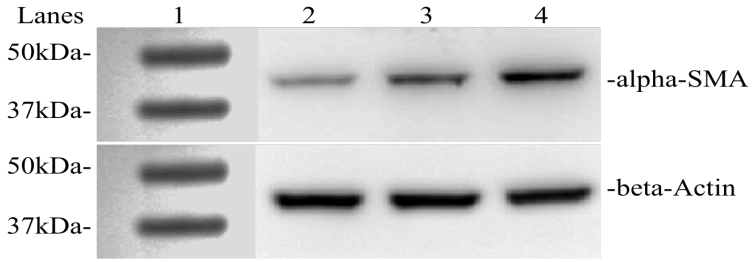

Figure 8: Western blot analysis of alpha-SMA expression in MRC5-hKLF4 cells following KLF4 over-expression in MRC5-hKLF4**.** Protein analysis of alpha-SMA expression in MRC5-hKLF4 cells following DOX exposure for 24 hours (Lane 2). Lane 1: Marker, Lane 2: 20μg whole cell lysate MRC5-hKLF4 cells following DOX exposure for 24 hours, Lane 3: 20μg whole cell lysate MRC5-hKLF4 cells without DOX exposure, Lane 4: 20μg whole cell lysate MRC5 cells exposed to DOX. Bottom row is the Beta-actin loading control.

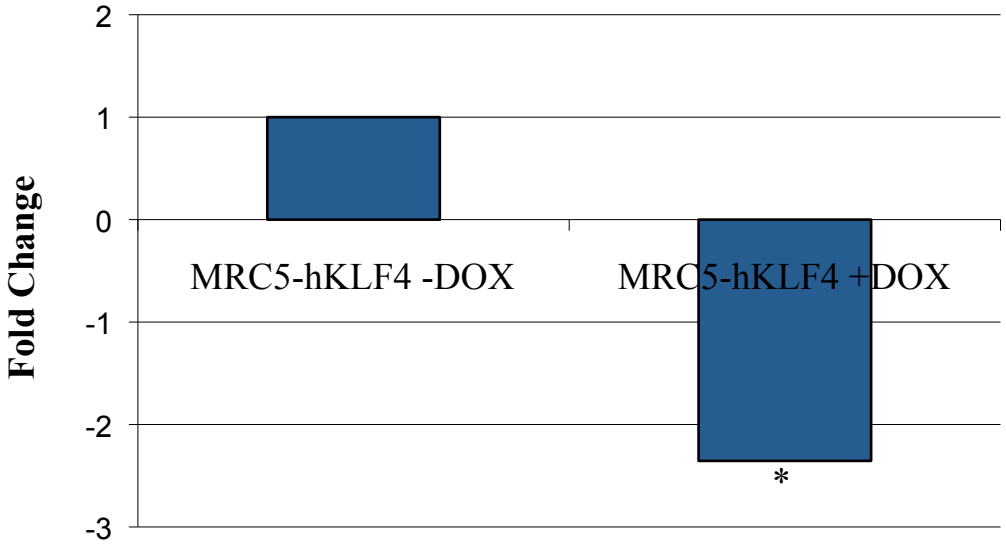

Figure 9: Bar graph of densitometry readings for relative alpha-SMA protein expression MRC5-hKLF4 cells following KLF4 induction for 24 hours. MRC5-hKLF4 cells were exposed to DOX for 24 hours to induce KLF4 over-expression (n=3). Negative control is MRC5-hKLF4 not exposed to DOX (n=3). Samples were normalized to beta-actin protein expression. * p value ≤ 0.05 as determined by paired t test.

Effect of KLF4 over-expression on NANOG gene expression:

To further evaluate the effect of KLF4 on differentiation and MET in MRC5 cells, the gene expression of NANOG was monitored following KLF4 over-expression. After KLF4 induction for 24 hours NANOG was observed to increase 1.95-fold (p-value=.0479) (Fig. 10).

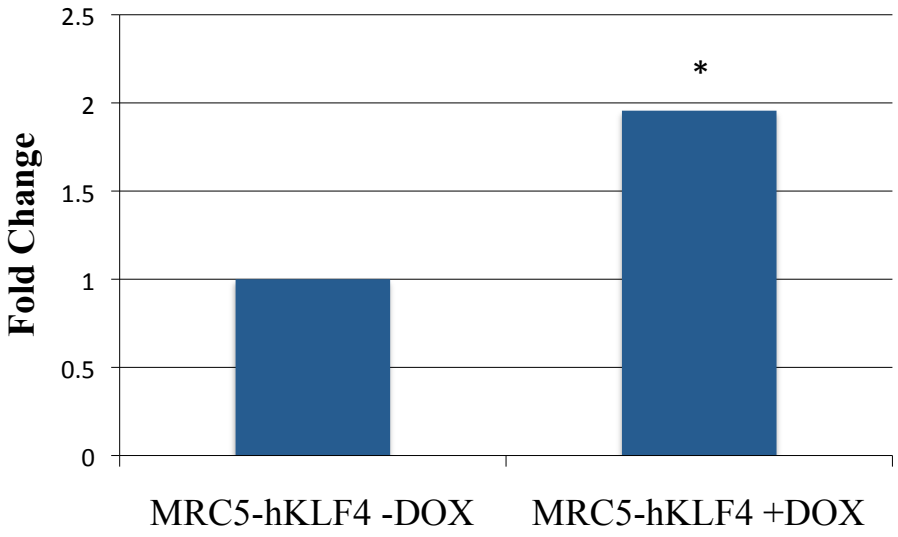

Figure 10: Fold change in gene expression of *NANOG* following KLF4 over-expression in MRC5-hKLF4. MRC5-hKLF4 cells were exposed to DOX for 24 hours and compared to negative control, MRC5-hKLF4 without DOX. * p value ≤ 0.05 as determined by paired t test.

Effect of KLF4 over-expression on proliferation markers:

Analysis of KLF4 over-expression on downstream proliferation markers, p21, p53, PCNA and cyclin D1 was also carried out on MRD5-hKLF4 cells. Expression of, p53, cyclin D1 and PCNA mRNA was decreased relative to MRC5-hKLF4 -DOX cells (Fig. 11). Specifically, p53 gene expression decreased 2.72-fold (p-value=.0476), cyclin D1 gene expression decreased 2.43-fold (p-value=.0029) and PCNA gene expression decreased 3.25-fold (p-value=0.0450) (Fig. 11). Expression of, p21 gene was found to be decreased 1.95-fold relative to MRC5-hKLF4 -DOX cells (p-value=.0012) (Fig. 11).

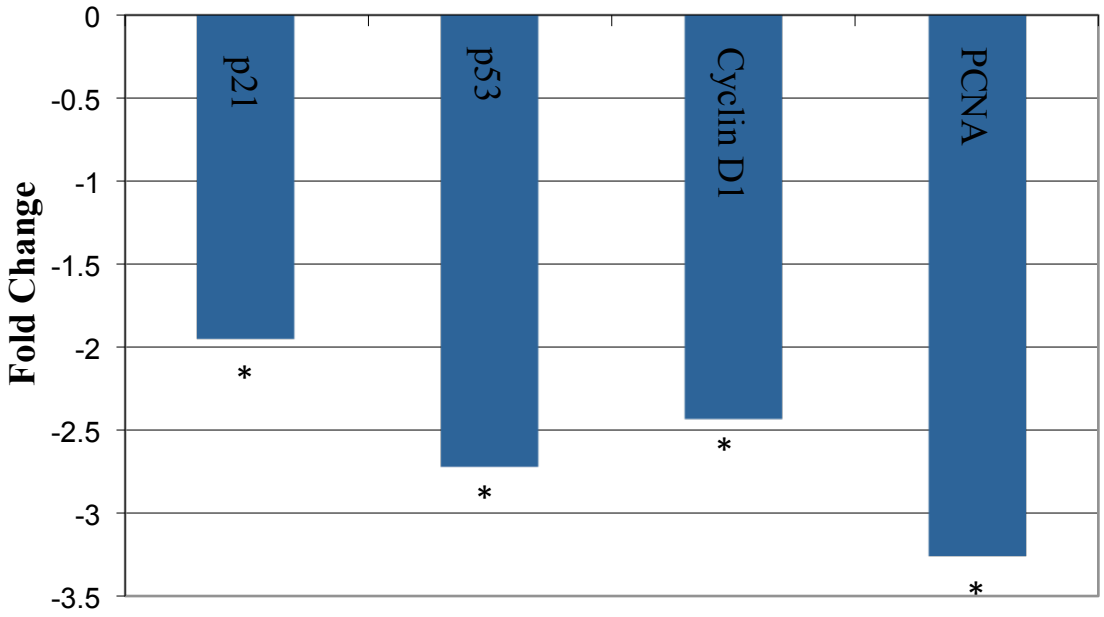

Figure 11: Fold change in mRNA of proliferation markers (p21, p53, cyclin D1 and PCNA) following KLF4 over-expression in MRC5-hKLF4 for 24 hours. MRC5-hKLF4 cells were exposed to DOX for 24 hours to induce KLF4 over-expression. Negative control is MRC5-hKLF4 not exposed to DOX. * denotes a p value ≤ 0.05 as determined by paired t test.

Effect of KLF4 over-expression on cell survival:

To investigate the effect of KLF4 over-expression on cellular survival cells were exposed to a lethal concentration (0.5mM) of hydrogen peroxide (H_2O_2) for 24 hours in the presence and absence of KLF4 induction by DOX. Cellular survival in the presence of over expression of KLF4 was not significantly different compared to cells without KLF4 induction (Fig. 12). MRC5 +DOX cells exposed H_2O_2 displayed 7.21% survival. MRC5-hKLF4 –DOX cells exposed to H_2O_2 displayed 6.7% survival (p-value=.1719), while MRC5-hKLF4 +DOX cells exposed to H_2O_2 displayed 6.9% survival (p-value=.2928) (Fig. 12).

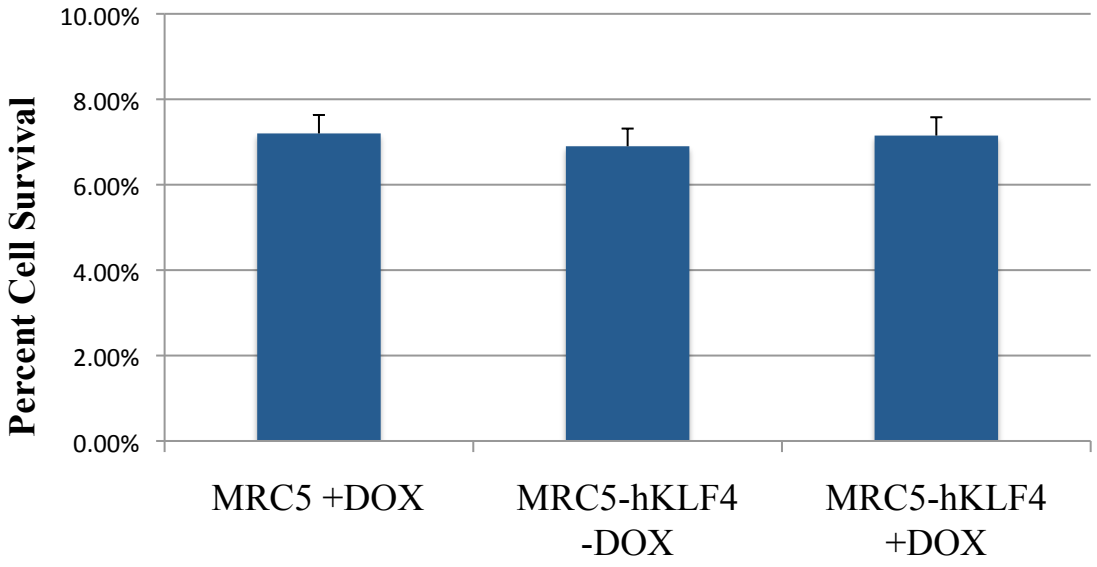

Figure 12: Percent cell survival following 0.5mM H_2O_2 exposure concomitant with KLF4 induction for 24 hours. MRC5 +DOX displayed 7.21% survival, MRC5-hKLF4 cells –DOX displayed 6.7% survival and MRC5-hKLF4 cells +DOX displayed 6.9% survival. No significant difference between samples was observed.

KLF4 localization in IPF tissue:

IHC analysis revealed elevated levels of KLF4 protein expression in IPF lung samples relative to the normal lung samples (Fig. 13 & 14). KLF4 positive staining is observed around the perimeter of the fibrotic foci and randomly throughout the parenchyma regions where advanced fibrosis is observed (Fig. 13 & 14). Proliferation marker PCNA positive staining was observed at higher levels in the IPF lung compared to the normal lung and was typically located around the perimeter of the fibrotic foci (Fig. 13 & 14). Dual staining demonstrated KLF4 and PCNA frequently co-localization around the perimeter of the fibrotic foci, however, this co-localization was not always observed (Fig. 13 & 14). Elevated expression levels of alpha-SMA protein were observed within the fibrotic foci relative to the parenchymal areas (Fig. 13 & 14). While, alpha-SMA positive staining in the normal lung is only observed in the vascular tissue (Fig. 15 & 16). KLF4 and alpha-SMA positive staining rarely co-localize in the IPF lung (Fig. 13 & 14). The normal parenchyma displayed no significant KLF4, alpha-SMA or PCNA staining (Fig. 15 & 16).

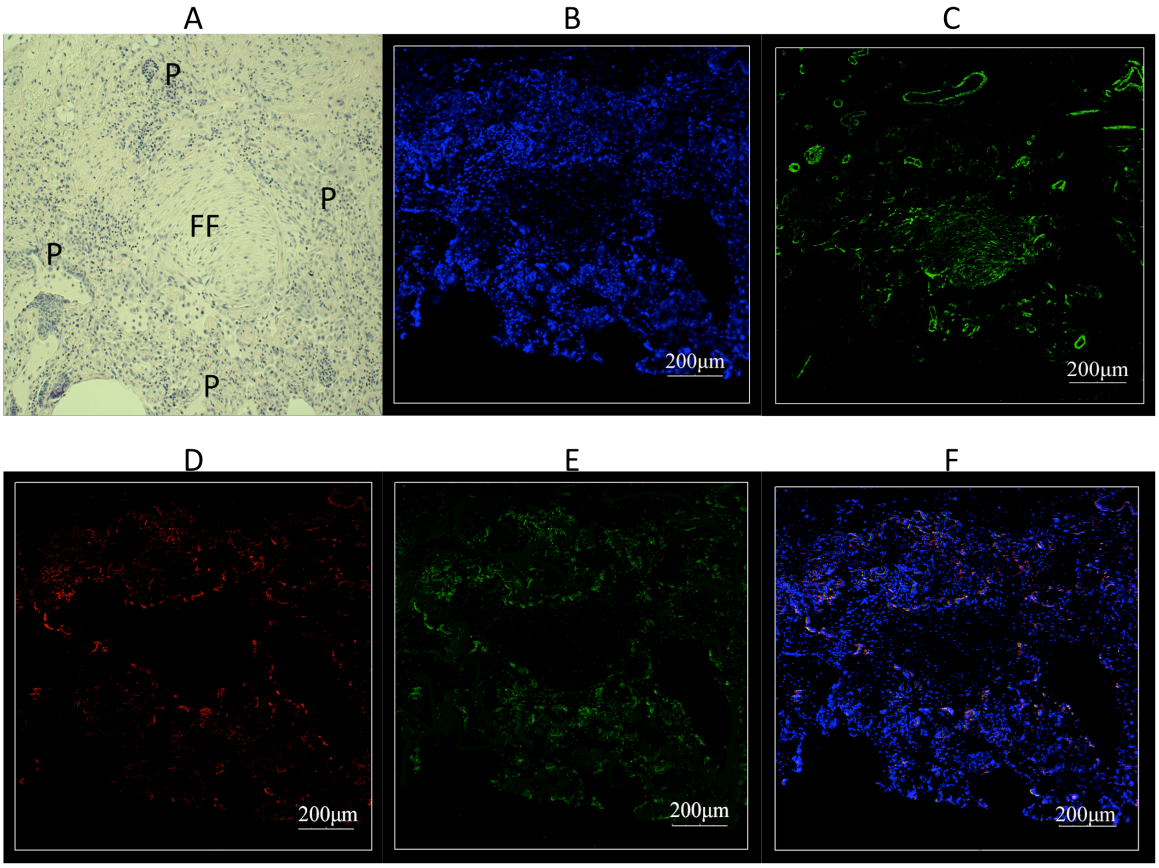

Figure 13: IHC analysis of a fibrotic foci (FF) in an IPF lung for expression of KLF4, PCNA and alpha-SMA proteins at 10x objective magnification. A: H&E of fibrotic foci. B: DAPI staining of corresponding fibrotic foci. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

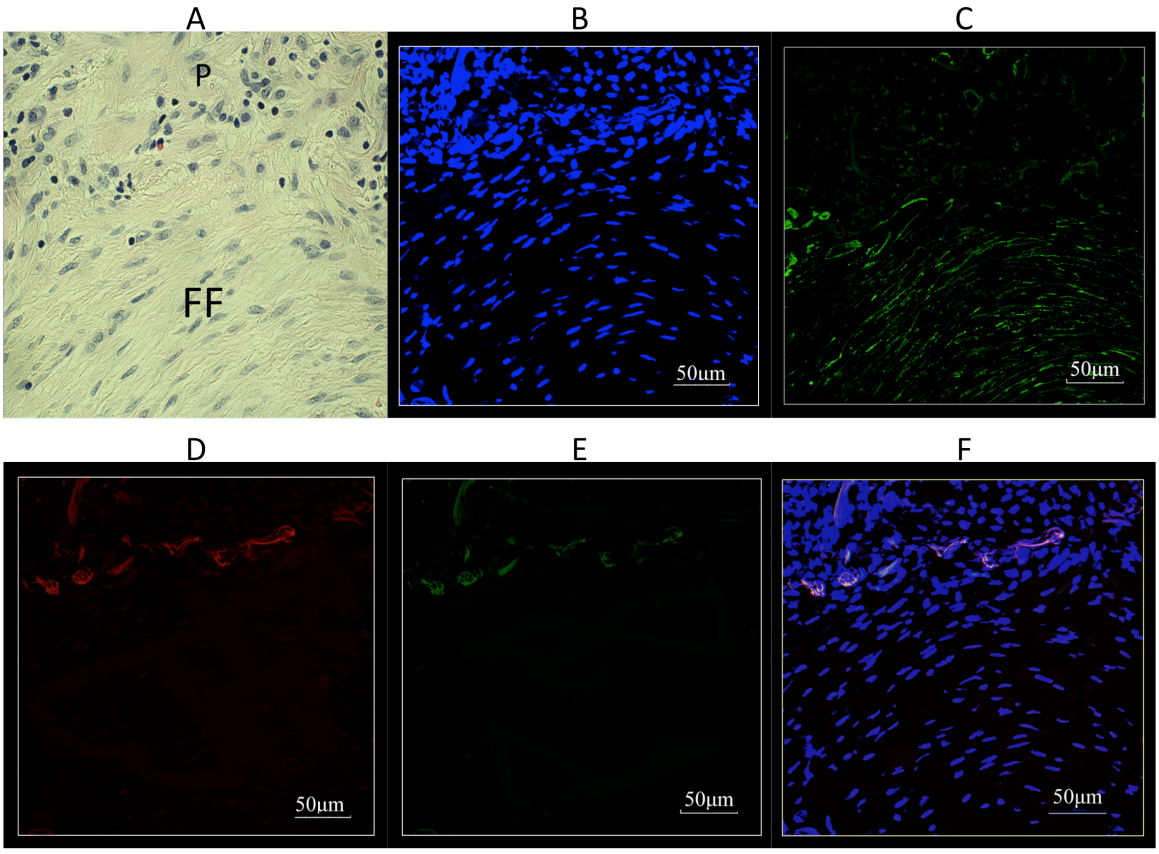

Figure 14: IHC analysis of the same fibrotic foci (FF) from Figure 13 (IPF lung) for expression of KLF4, PCNA and alpha-SMA proteins at 40x objective magnification. A: H&E of fibrotic foci. B: DAPI staining of corresponding fibrotic foci. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

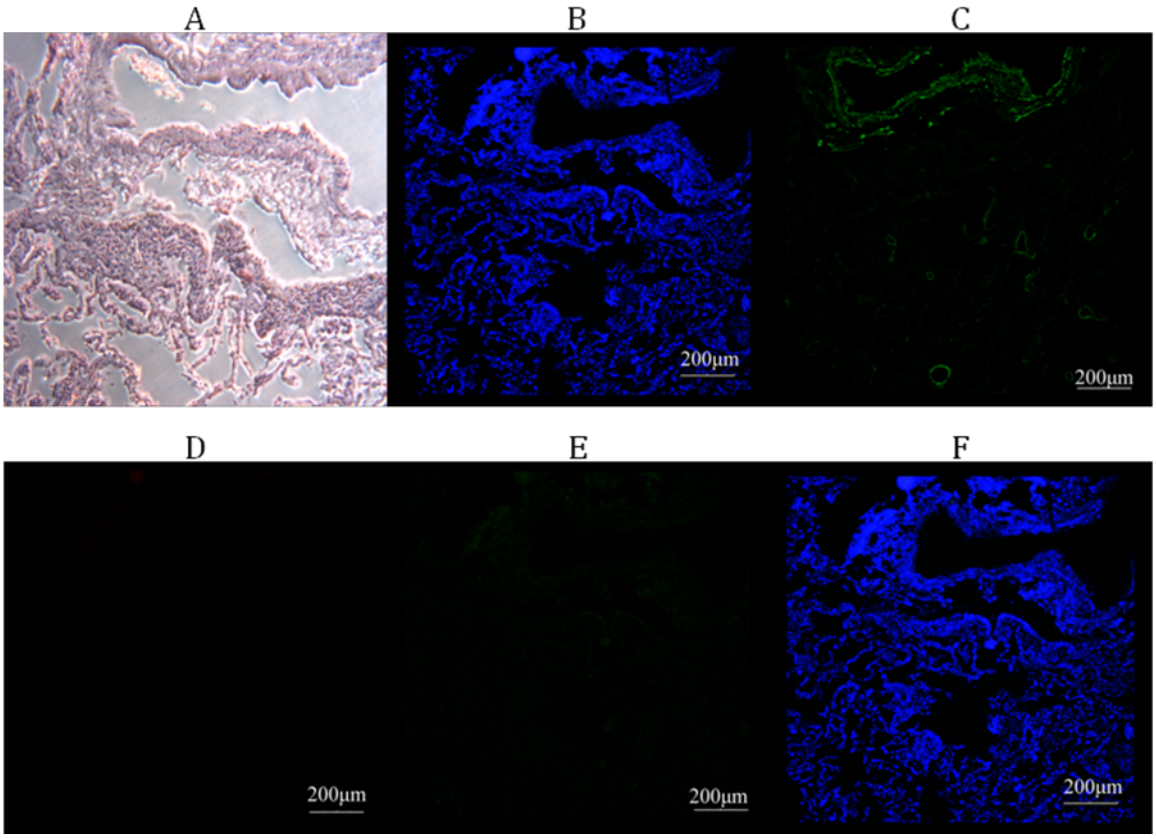

Figure 15: IHC analysis in a normal lung for expression of KLF4, PCNA and alpha-SMA proteins at 10x objective magnification. A: H&E of normal lung tissue. B: DAPI staining of the corresponding locale. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

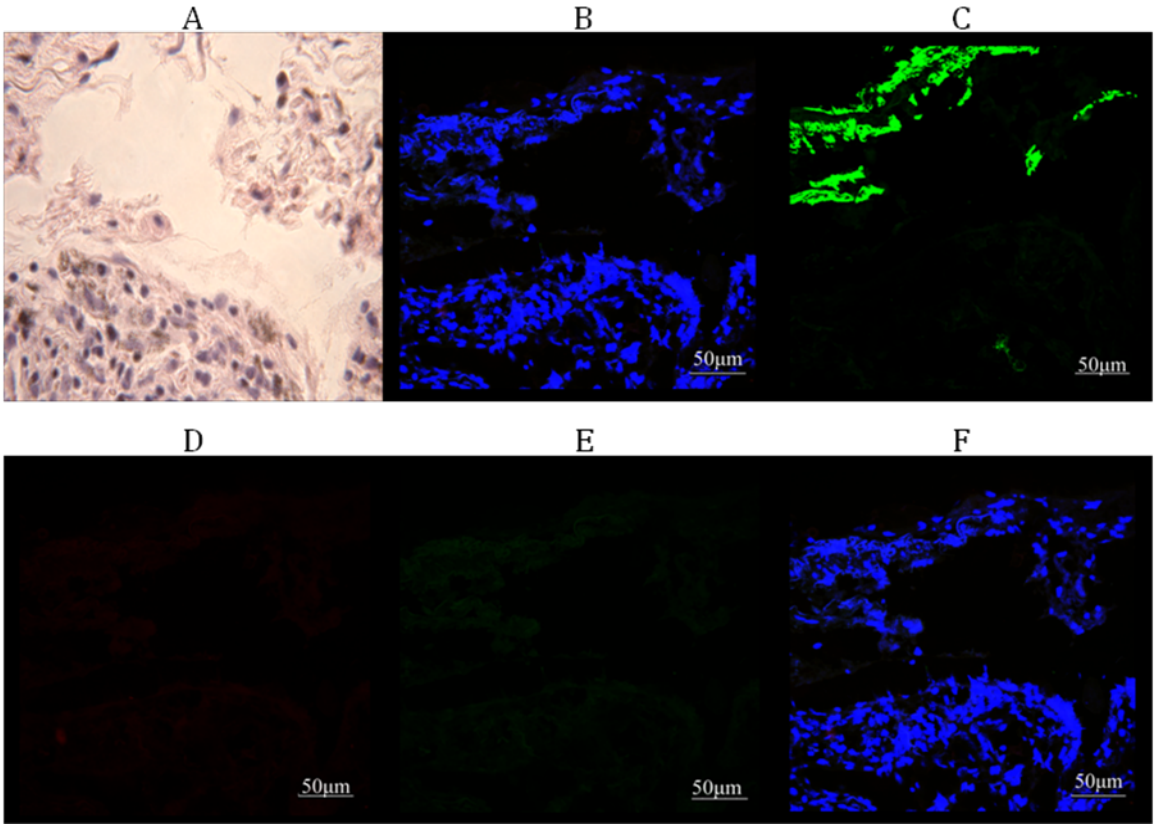

Figure 16: IHC analysis of the same location from Figure 15 (normal lung) for expression of KLF4, PCNA and alpha-SMA proteins at 40x objective magnification. A: H&E of normal lung tissue. B: DAPI staining of the corresponding locale. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

CHAPTER 4: Discussion

The overall aim of this study was to investigate the potential role of KLF4 over-expression in the pathogenesis of IPF. KLF4 plays a role in regulating a number of cellular processes including proliferation, apoptosis, cellular homeostasis and differentiation of various tissues such as the skin, colon, and eye (Segre 1999; Katz 2002; McConnell 2007; Chen 2008; Evans 2008). Paradoxically, KLF4 is thought to act as a tumor suppressor gene in colon (Dang 2003; Zhao 2004), gastric (Wei 2005; Kanai 2006) and lung cancer (Hu 2009; Zhou 2010) and as an oncogene in breast cancer (Yu 2011) and cutaneous squamous cell carcinoma (SCC) (Huang 2005; Chen 2008). Based on the oncogenic properties it was hypothesized that the KLF4 over-expression observed in the IPF fibroblasts population may contribute to the hyper-proliferative anti-apoptotic phenotype observed within this population. Initially we investigated the localization of KLF4 expression in both normal and IPF lung tissue slices to gain insight into the potential role of KLF4 within the context of IPF. To test the translational hypothesis that KLF4 over-expression contributes to the pathogenesis of IPF, KLF4 was over-expressed by an inducible lenti-viral system in the fetal human fibroblast cell line, MRC5. We then focused on the effect of KLF4 over-expression on the activation status and proliferative state of MRC5 cells based on KLF4's ability to regulated alpha-SMA expression and the cyclins, p21 and p53, respectively.

KLF4 Localization in IPF:

To localize KLF4 in IPF lung tissue, dual IHC analyses were performed using a combination of KLF4 and alpha SMA or KLF4 and PCNA antibodies. Dual IHC staining for alpha-SMA and KLF4 in the IPF tissue samples demonstrated that KLF4 and alpha-SMA very rarely co-localize (Figure 13 & 14). Alpha-SMA staining was predominantly observed within the fibrotic foci and dispersed sparingly throughout the parenchyma (Figure 13 & 14). These data are in agreement with previous observations [Chhina, PhD Dissertation, GMU, 2010] (Yamada 2008). KLF4 staining was more in evidence on the perimeter of the fibrotic foci and sparsely throughout the parenchyma (Figure 13 & 14). This lack of general co-localization agrees with the theory that KLF4 regulates and inhibits the expression of alpha-SMA (Adam 2000; Liu 2003; Liu 2005; Hu 2007). Small patches of cells throughout the parenchyma did, however, stain positive for both alpha-SMA and KLF4. This suggests that mixed populations of fibroblast like cells, potentially at various stages of development and/or activation, are present throughout the IPF lung. In normal tissue KLF4 staining was not significantly evident, in addition, alpha-SMA staining was absent through out the normal lung except for within vascular tissue, which concurs with previous data (Yamada 2008).

Dual IHC analysis for PCNA and KLF4 revealed that these proteins frequently co-localized on the perimeter of the fibrotic foci and sparsely throughout the parenchyma (Figure 13 & 14). This pattern of PCNA staining is in agreement with previous data,

which demonstrates that this protein is absent from the fibrotic foci but present on the peripheries of these foci and throughout the IPF parenchyma [Chhina, PhD Dissertation, GMU, 2010]. The co-localization of PCNA and KLF4 is of particular interest due to the fact that KLF4 is also observed to co-localize with PCNA in cutaneous SCC and is thought to aid in tumor development and metastasis of cutaneous SCC (Huang 2005; Chen 2008). If KLF4 and PCNA are co-localizing within the same cell, then it is possible that KLF4 is involved in the pathogenesis of IPF, potentially via induction of a hyper-proliferative state. These KLF4 over-expressing cells may have by-passed the cell cycle inhibitory mechanism of p21 resulting in unchecked proliferation (Rowland 2005; Rowland 2006). However, to accurately determine if true cellular co-localization of both KLF4 and PCNA is occurring further experimentation via Fluorescence Resonance Energy Transfer (FRET) analysis would need to be carried out.

The Effect of KLF4 over-expression on fibroblast activation markers:

KLF4 over-expression can lead to both inhibition and induction of the fibroblast activation marker, alpha-SMA (Liu 2003; Liu 2005; Hu 2007; Wang 2008; Li 2010). Inhibition of alpha-SMA expression can occur in a multitude of ways (Adam 2000; Liu 2003; Liu 2005; Hu 2007). However, the mechanism by which KLF4 up-regulates alpha-SMA expression is not currently understood (Wang 2008; Li 2010). From our data demonstrating KLF4 over-expression in MRC5 cells we report a concurrent down-regulation of alpha-SMA gene and protein expression (Figure 7, 8 & 9). The mechanism by which this down-regulation of alpha-SMA occurs in these cells has not yet been

determined. However it is most likely through direct or indirect interference with expression at the promoter level, potentially though interaction with TGF-beta intermediary Smad3 as previously reported to occur in rat fibroblasts (Hu 2007). This may be one of many functions of KLF4 in IPF fibroblasts, which is further corroborated by the IHC observations. These data taken together with previous findings supports the notion that the environment as well as the cell type significantly influences the function of KLF4, a theory that is also gaining traction for IPF. Given this role of KLF4 in MRC5 cells, performing these studies out in primary IPF fibroblast to gain more insight into the over-expression of KLF4 in IPF.

Further investigations into the effects of KLF4 over-expression on other markers of fibroblast activation and components of the IPF ECM, Collagen 1A1 and fibronectin, were then carried out. KLF4 has not previously been associated with the over-expression of Collagen 1A1, however, KLF4 mediates 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC) induced collagen 8A1 expression in VSMC (Cherepanova 2009). Collagen 1A1 was found to be significantly up-regulated (3-fold) following KLF4 over-expression (Figure 7). The KLF4 induced over-expression observed in this study is supportive of a role for KLF4 in IPF fibroblast and their excessive ECM production (Figure 7) (Raghu 1985).

The data presented here demonstrate that KLF4 over-expression influences markers of fibroblast activation *in vitro*. However, with all *in vitro* studies, extrapolation to the *in*

vivo environment should be carried out with caution, especially in cases such as IPF where the *in vitro* environment differs considerably from the *in vivo* environment. Additionally, there are potentially multiple origins of the IPF fibroblasts and MRC5s, which are fetal in origin, may not respond to KLF4 over-expression in the same manner as adult primary IPF fibroblasts. To provide relative context, KLF4 should be over-expressed in primary IPF and normal fibroblasts. Additionally, *in vivo* localization of Collagen 1A1 in IPF tissue would support the hypothesis that KLF4 plays a role in the excessive ECM deposition observed in IPF (Raghu 1985).

The Effect of KLF4 over-expression on NANOG gene expression:

NANOG expression in concert with other factors such as c-Myc, Oct4 and Sox2 is thought to function in establishing ES cell identity and pluripotency from fibroblasts (Zhao 2008; Wang 2010; Zhang 2010). KLF4 has been reported to induce the expression of *NANOG*, both in the presence and absence of other reprogramming transcription factors Oct4 and Sox2 (Wei 2009; Zhang 2010). We also observed an increase in *NANOG* gene expression following KLF4 over-expression in MRC5-hKLF4 cells, which could indicate a tendency toward a dedifferentiation state, or could also signify mesenchymal-to-epithelial transition (MET) (Wang 2010). MET, which is a key pathway in the generation of iPS cells from fibroblasts (Li 2010; Polo 2010; Wang 2010) and can also be induced via KLF4 over-expression in corroboration with Sox2, Oct4 and c-Myc (Li 2010). Knockdowns of KLF4 have also been associated with induction of a fibroblastic like morphology concomitant with a loss of E-cadherin and an appearance of

N-cadherin (Yori 2010). This phenomenon, dubbed "cadherin switching" is often associated with EMT (Maeda 2005) and a more invasive phenotype in cancer (Hazan 2004). Activating the MET pathway in the IPF lung could lead to a decrease in the overall fibroblast population, thereby countering IPF pathogenesis. We postulate that may be the role of KLF4 in IPF - to again counter the pathogenesis of IPF. However, this would need to be supported though experimentation in primary IPF fibroblasts. Additionally, localization of *NANOG* and other markers for MET/EMT would need to be investigated *in vivo* for this theory to be advanced.

The Effect of KLF4 over-expression on fibroblasts proliferation:

KLF4 is known to induce expression of p21 (Zhang 2000; Rowland 2005) and repress cyclin D1 (Shie 2000), cyclin B1 (Yoon 2004) and cyclin E expression (Yoon 2005), resulting in cell-cycle arrest. In this study KLF4 over-expression in MRC5s resulted in the down-regulation of a series of proliferation markers (Figure 11). This is in agreement with a number of previous studies documenting KLF4's ability to regulate the cell cycle. Unexpectedly, a decrease in expression of the cell cycle inhibitor and checkpoint protein, p21, was observed (figure 11). Previous studies have shown that the loss of p21 expression concomitant with KLF4 over-expression promotes proliferation and a loss of contact inhibition in MEFs, a similar phenotype to the rapidly proliferating apoptosis resistant IPF fibroblast (Rowland 2005). The concomitant down-regulation of p53 and p21 observed in this study could also suggest induction of an oncogenic phenotype capable of proliferating in an unchecked manner due to decreased p21 and

resistant to apoptosis due to decreased p53 expression (Rowland 2005; Rowland 2006). To investigate if the concomitant suppression of p21 and p53 in the MRC5-hKLF4 cells resulted in an apoptosis resistant phenotype, these cells were exposure to a lethal concentration of apoptosis-inducing hydrogen peroxide. No significant increase in survival was observed for these cells, however (Figure 12).

KLF4 over-expression in MRC5-hKLF4 cells resulted in an apparent non-proliferative phenotype, which implicates KLF4 as a functional tumor suppressor in MRC5 cells and potentially other fibroblasts. Nevertheless, the down-regulation of p21 may indicate a pro-proliferative state given the right milieu of ECM growth factors such as TGF-beta. Moreover, a heterogeneous population of fibroblasts likely exists in the IPF lung, as previously hypothesized [Chhina, PhD Dissertation, GMU, 2010] (Uhal 1998). The KLF4-expressing IPF fibroblasts may be a subpopulation that are terminally differentiated, collagen 1A1-expressing and non-motile, lacking alpha-SMA. Additionally, these KLF4-expressing fibroblasts might be susceptible to external growth factors, as a consequence of low p21 levels. Therefore, KLF4 may promote unchecked proliferation in myofibroblasts as observed in MEFs (Rowland 2005) given the right environment, as the context is influential in determining the function of KLF4.

KLF4s and the TGF-beta pathway in IPF:

Other potential roles for KLF4 in IPF exist within the context of TGF-beta signaling. As mentioned previously, TGF-beta is one of the most important disease-orientated signaling mechanisms in IPF. In IPF, KLF4 is localized on the perimeter of the fibrotic foci (Figure 13 & 14) where there it may be acting to fight against TGF-beta induced EMT of alveolar epithelial cells. In particular, KLF4 may act as a natural counter balance to TGF-beta induced EMT, by induction of the opposite MET pathway and inducing the expression of E-caherin (LaGamba 2005; Li 2010; Wang 2010; Yori 2010). In further support of this theory, KLF4 can also interact with the TGF-beta signal-transducing molecule Smad3 and inhibit TGF-beta induced alpha-SMA expression and myofibroblast differentiation (Hu 2007). TGF-beta can inhibit KLF4 protein function, thereby preventing KLF4 from blocking TGF-beta induced smooth muscle gene expression (Kawai-Kowase 2009; Hu 2010). Taken together KLF4 and TGF-beta appear to act as the yin and yang of the EMT and MET control mechanisms in the IPF lung. KLF4 may be acting to inhibit EMT of the alveolar epithelial cells, one potential source of myofibroblasts and inhibiting alpha SMA expression, thereby acting as a counter to TGF-beta induced pathogenesis of IPF.

KLF4 and the Wnt pathway in IPF:

In addition to the TGF-beta pathway, the Wnt pathway is believed to influence EMT (von Gise 2011), and recently, studies have revealed that IPF fibroblasts display aberrant Wnt signaling (Chilosi 2003; Yang 2007; Emblom-Callahan 2010). Specifically, in IPF fibroblasts compared to normal fibroblast, disheveled homolog 1 (DVL 1) is down-regulated 1.5-fold while secreted Frizzled Related Protein 2 (sFRP2) and prickle homolog 1 (PRICKLE1) are up-regulated 3-fold and 1.8-fold, respectively (Emblom-Callahan 2010). Furthermore, sFRP2 has recently been implicated in cardiac fibrosis (Kobayashi 2009) and beta-catenin is observed to localize in the fibroblastic foci of IPF *in vivo* (Chilosi 2003). KLF4 influences the Wnt pathway through direct interaction with beta-catenin and can inhibit p300/CBP-mediated beta-catenin acetylation on Wnt target genes (Evans 2009). Additionally, KLF4 can inhibit beta-catenin mediated gene expression (Zhang 2006). The Wnt pathway has been implicated in promoting EMT in IPF. Specifically, a beta-catenin/Smad2 transcriptional complex is thought help initiate EMT in AECs (Kim 2009). However, the exact mechanism by which this occurs remains to be elucidated. KLF4 may interfere with the formation of the beta-catenin /Smad2 complex, thereby preventing EMT and countering IPF pathogenesis. This may occur through direct interaction with the beta-catenin protein or Smad2. This is of great significance considering the role of EMT and Wnt signaling in IPF. Further investigations are warranted and should explore the relationship between KLF4 and the Wnt pathway in the context of IPF.

KLF4 and INF-gamma in IPF:

INF-gamma is also a key mediator of antagonizing the TGF-beta pathway, resulting in a reduction of proliferation, differentiation and collagen synthesis (Hauber 2010). INF-gamma treatment of fibrosis in the bleomycin-induced IPF mouse model also demonstrated a decrease in TGF-beta, Collagen I and III mRNA. Furthermore, *in vitro* studies with primary IPF and normal fibroblast demonstrated a decrease in collagen mRNA following IFN-gamma exposure (Narayanan 1992). These studies along with other reported anti-fibrotic behaviors prompted the INSPIRE Trial: A Study of Interferon Gamma-1b for Idiopathic Pulmonary Fibrosis (IPF). However this study was terminated in 2009 due to the test drug showing lack of benefit at interim analysis (King 2009). Additionally, further investigations with IFN-gamma showed no alteration of TGF-beta or CTGF mRNA levels in IPF whole lung analysis (Strieter 2004; Tzortzaki 2007). Interestingly, IFN-gamma is known to regulate the gene expression of KLF4 in mouse fibroblasts (Chen 2002; Feinberg 2005) and considering the increased expression of KLF4 observed in the IPF lung, perhaps treatment with INF-gamma exacerbated a potential pathogenic role of KLF4. Further investigations of the effect of INF-gamma on KLF4 gene expression and its downstream components in IPF cells would shed light on this issue.

KLF4 and apoptosis in IPF:

The influence of apoptosis resistance on the IPF fibroblasts population is a highly controversial topic. While some suggest they are more apoptosis resistant (Thannickal 2006; Fattman 2008), others, on the contrary, suggest IPF fibroblasts exhibit an increased rate of apoptosis derived from lung tissue explants (Ramos 2001). Nonetheless, we have found that a wide array of apoptosis genes are altered in IPF fibroblasts, many of which are pro-apoptotic in nature (Emblom-Callahan 2010). KLF4 is known to play a role in cell survival via inhibition of apoptosis, and this may be a role for KLF4 in IPF. Apoptosis resistance following KLF4 over-expression may occur when p53 expression is suppressed. Moreover, if p53 repression is concomitant with the by-pass of p21 this could lead to apoptosis-resistant and hyper-proliferative cells as is believed to exist in IPF. However, when KLF4 was over-expressed in the fetal MRC5 cell line resistance to apoptosis was not observed (Figure 11 & 12). Given these data and the fact that p21 expression is up-regulated in IPF fibroblasts (Emblom-Callahan 2007) suggest that this mechanism may not be at play in IPF. However as we have stated previously, the IPF fibroblast population is made up of multiple populations, and those over-expressing KLF4 appear to be a subset. In that same vein, this subpopulation may have altered p21 expression levels, thereby, inducing an anti-apoptotic hyper-proliferative phenotype.

Conclusions:

Based on these results we conclude that KLF4 supports terminal differentiation by inhibiting proliferation and alpha-SMA expression and promoting ECM production in MRC5 cells *in vitro*. In partial support of this conclusion the *in vitro* data demonstrated that KLF4 and alpha-SMA rarely co-localize *in vivo* in IPF. However, in the *in vitro* data demonstrated PCNA and KLF4 co-localize on the edge of the fibrotic foci and through out the parenchyma of the IPF lung (Figure 12 & 13). Therefore, KLF4 may promote proliferation of a subpopulation of IPF fibroblasts as observed *in vivo*. Furthermore, KLF4-induced ECM production in MCR5 cells and its over-expression my also support ECM production in IPF fibroblasts, thereby contributing to IPF pathogenesis. We hypothesis that the IPF lung is composed of a heterogeneous population of fibroblasts at varying stages of differentiation/activation and proliferation as previously hypothesized and that a subpopulation of these cells express KLF4 [Chhina, PhD Dissertation, GMU, 2010] (Uhal 1998). Additionally, KLF4 is a highly context-dependent protein and its actions in IPF fibroblasts may differ greatly from what is observed in MRC5 cells, which is a normal fetal lung fibroblast cell line. Therefore, *in vitro* studies in primary IPF fibroblasts need be explored in order to characterize the role of KLF4 in the context of IPF. Furthermore, this study highlights the significant difference between *in vitro* models and the *in vivo* environment.

Many questions arise from our findings, which justify further characterization of KLF4's role in IPF. While the *in vivo* and *in vitro* data do not correlate exactly the data provides

evidence that KLF4 may be acting in a pathogenic manner either by promoting proliferation or inducing ECM production. Another potential role is that KLF4 may be acting to counter IPF pathogenesis especially given its interaction with TGF-beta and its role in the MET and EMT pathways. It is clearly that KLF4 is a significant factor in cellular homeostasis and its over-expression in IPF is potentially significant; however, its exact role is yet to be elucidated.

Future Directions:

Currently, there is no effective therapy for IPF, which is a fueled by the incomplete understanding of the disease pathogenesis, in addition to the lack of adequate disease models. In order to rectify this problem we must advance our understanding of IPF and the roles of significant transcription factors such as KLF4. To better understand KLF4 in the true context of IPF, over-expression in primary IPF fibroblasts should be explored. Furthermore, down-stream targets in addition to proteins known to interact with KLF4 should be localized in IPF tissue in light of the theory that multiple subpopulations of fibroblasts exist in the IPF lung. KLF4 interacts with many pathways and signaling mechanisms that participate in IPF, such as the Wnt pathway and the TGF-beta pathway. KLF4 also up-regulates the pluripotency protein NANOG and is also involved in the regulation of EMT and MET. Each of these pathways and proteins are significant players in IPF pathogenesis and their link with KLF4 suggests a significant role for this transcription factor in IPF. Further defining the role of KLF4 in IPF using primary cell

culture may illuminate the mechanism(s) of its role in protection and/or pathogenesis in IPF and subsequently lead to the development of an efficacious therapy or cure.

APPENDIX A: Supplemental Data

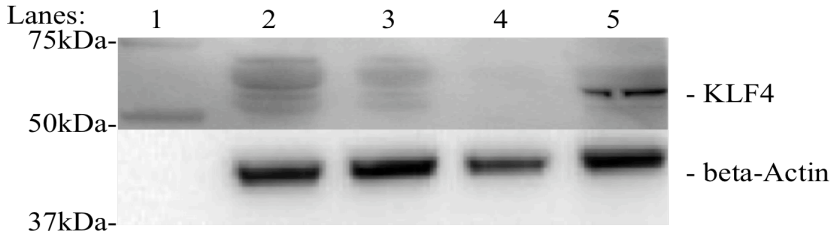

Figure A1: Western blot (#2) analysis of KLF4 protein expression in MRC5-hKLF4 cells following 24 hours DOX exposure. Lane 1: Marker, Lane 2: 10µg whole cell lysate MRC5-hKLF4 cells following DOX exposure for 24 hours, Lane 3: 10µg whole cell lysate MRC5-hKLF4 cells without DOX exposure, Lane 4: 10µg whole cell lysate MRC5 cells exposed to DOX, Lane 5: 10µg whole cell lysate of 293t cells, positive control for KLF4 protein expression. Bottom row is the Beta-actin loading control.

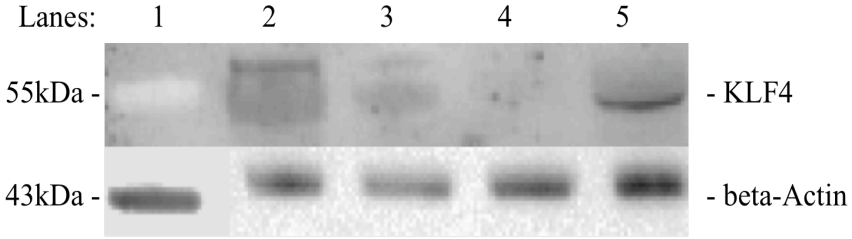

Figure A2: Western blot (#3) analysis of KLF4 protein expression in MRC5-hKLF4 cells following 24 hours DOX exposure. Lane 1: Marker, Lane 2: 10µg whole cell lysate MRC5-hKLF4 cells following DOX exposure for 24 hours, Lane 3: 10µg whole cell lysate MRC5-hKLF4 cells without DOX exposure, Lane 4: 10µg whole cell lysate MRC5 cells exposed to DOX, Lane 5: 10µg whole cell lysate of 293t cells, positive control for KLF4 protein expression. Bottom row is the Beta-actin loading control.

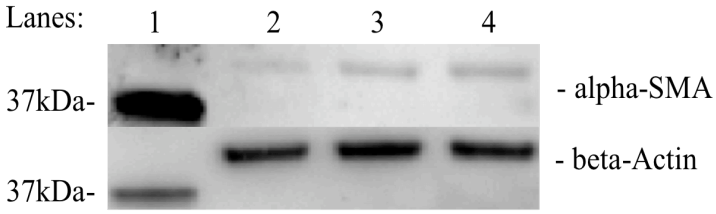

Figure A3: Western blot (#2) analysis of alpha-SMA protein expression in MRC5-hKLF4 cells following KLF4 over-expression in MRC5-hKLF4**.** Lane 1: Marker, Lane 2: 20μg whole cell lysate MRC5-hKLF4 cells following DOX exposure for 24 hours, Lane 3: 20μg whole cell lysate MRC5-hKLF4 cells without DOX exposure, Lane 4: 20μg whole cell lysate MRC5 cells exposed to DOX. Bottom row is the Beta-actin loading control.

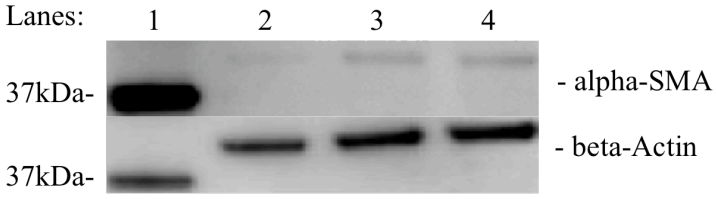

Figure A4: Western blot (#3) analysis of alpha-SMA protein expression in MRC5-hKLF4 cells following KLF4 over-expression in MRC5-hKLF4**.** Lane 1: Marker, Lane 2: 10μg whole cell lysate MRC5-hKLF4 cells following DOX exposure for 24 hours, Lane 3: 10μg whole cell lysate MRC5-hKLF4 cells without DOX exposure, Lane 4: 10μg whole cell lysate MRC5 cells exposed to DOX. Bottom row is the Beta-actin loading control.

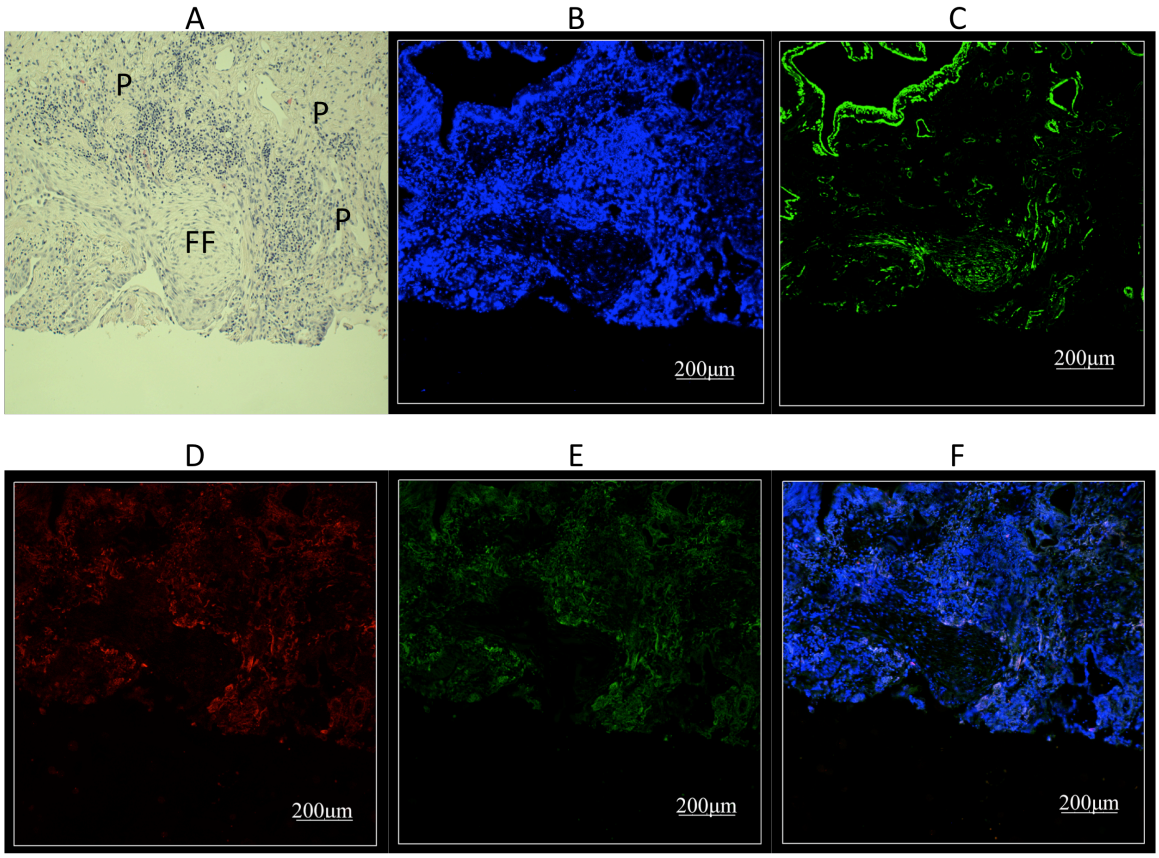

Figure A5: IHC analysis of a fibrotic foci (FF) in an IPF lung for expression of KLF4, PCNA and alpha-SMA proteins at 10x objective magnification. A: H&E of fibrotic foci. B: DAPI staining of corresponding fibrotic foci. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

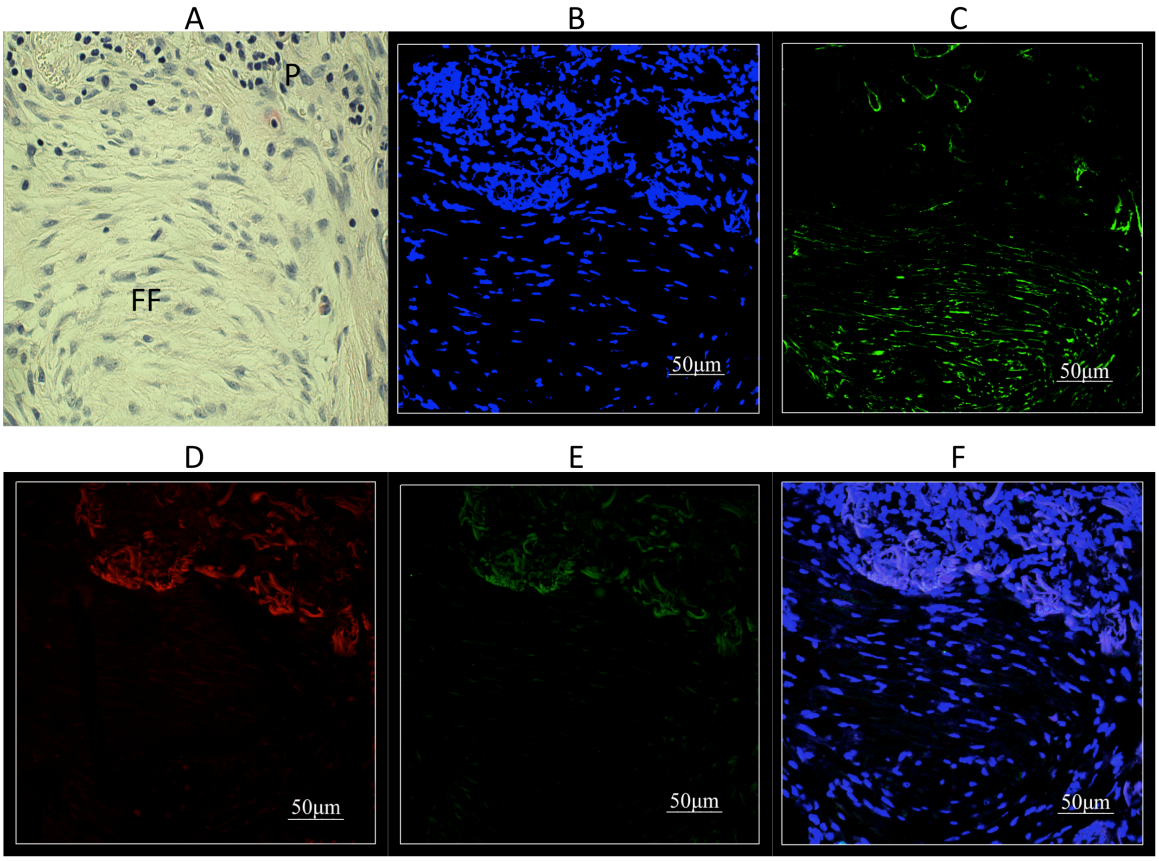

Figure A6: IHC analysis of the same fibrotic foci (FF) from Figure A5 (IPF lung) for expression of KLF4, PCNA and alpha-SMA proteins at 40x objective magnification. A: H&E of fibrotic foci. B: DAPI staining of corresponding fibrotic foci. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

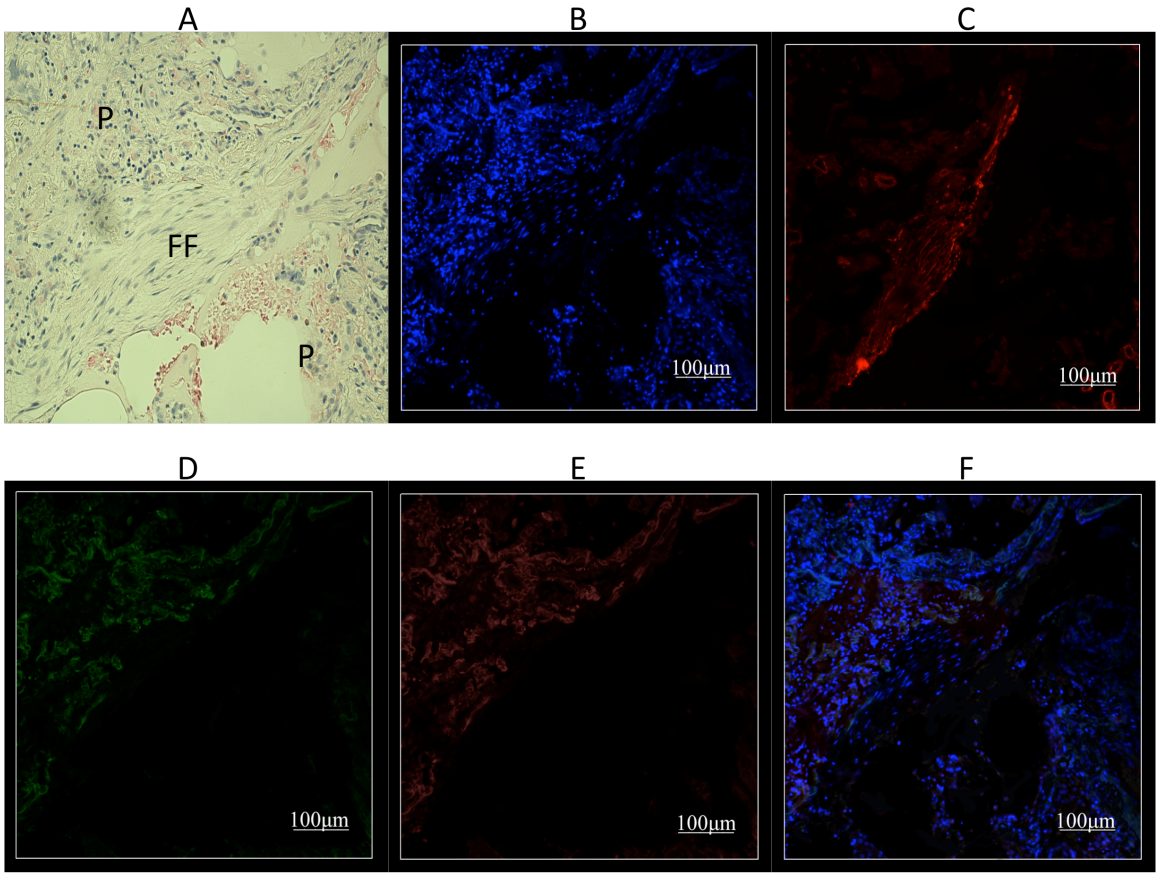

Figure A7: IHC analysis of a fibrotic foci (FF) in an IPF lung for expression of KLF4, PCNA and alpha-SMA proteins at 20x objective magnification. A: H&E of fibrotic foci. B: DAPI staining of corresponding fibrotic foci. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

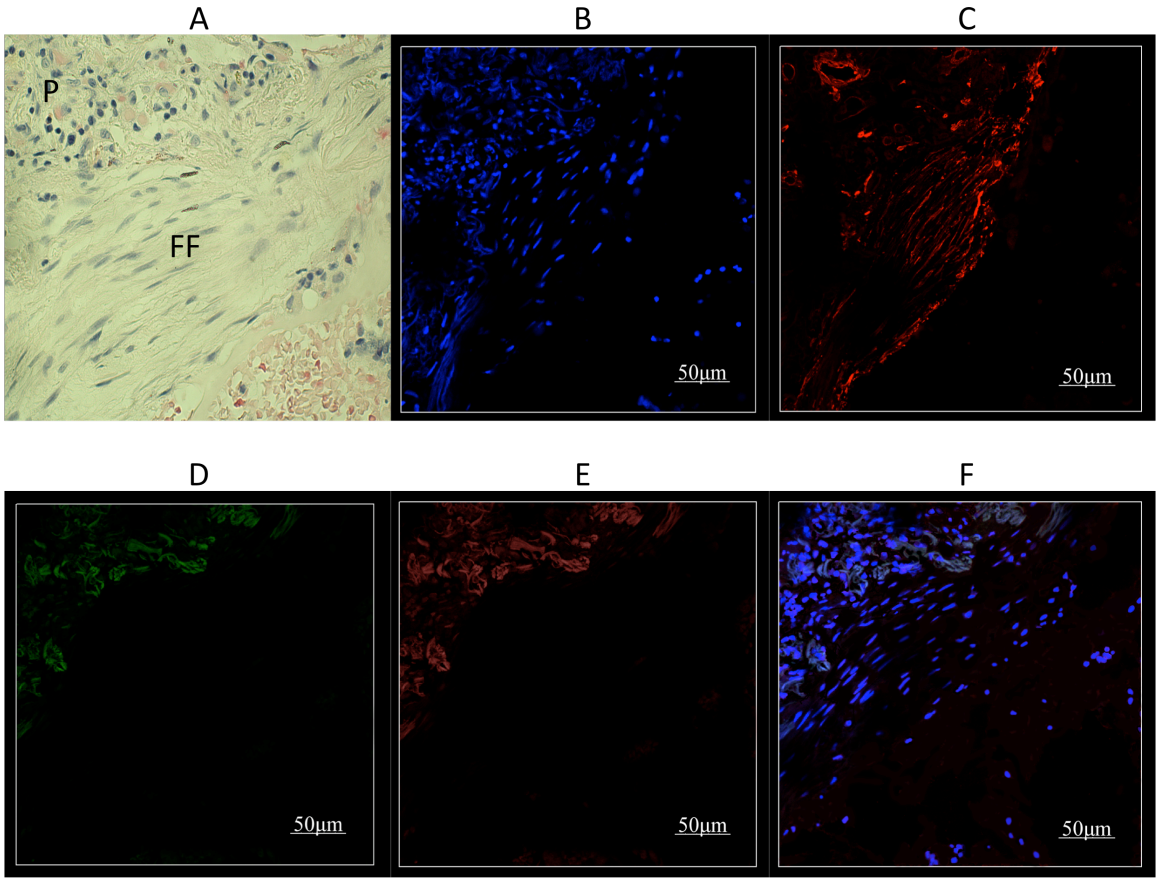

Figure A8: IHC analysis the same fibrotic foci (FF) from Figure A7 (IPF lung) for expression of KLF4, PCNA and alpha-SMA proteins at 40x objective magnification. A: H&E of fibrotic foci. B: DAPI staining of corresponding fibrotic foci. C: Red fluorescent staining of is representative of alpha-SMA localization. D: Green fluorescent staining is representative of KLF4 localization E: Red fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (red) and KLF4 (green) with white representative of co-localization.

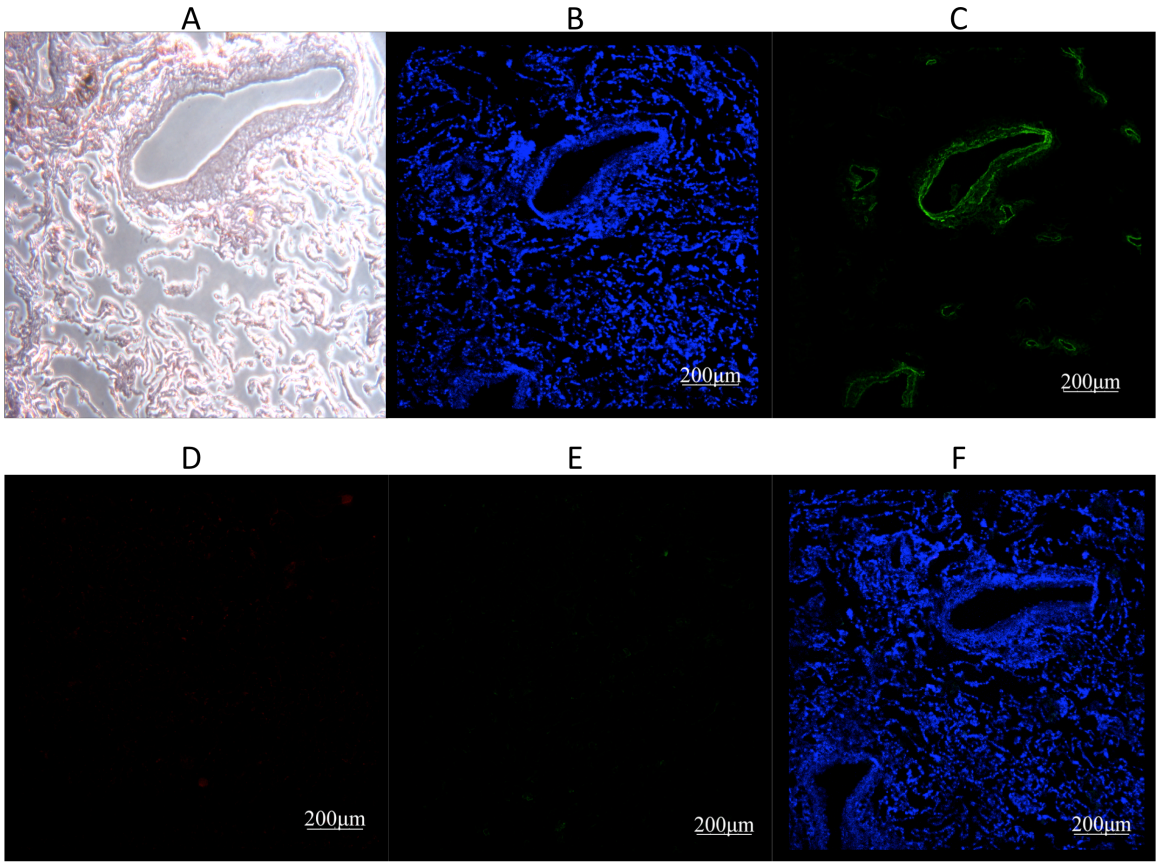

Figure A9: IHC analysis of a normal lung for expression of KLF4, PCNA and alpha-SMA proteins at 10x objective magnification. A: H&E of normal lung tissue. B: DAPI staining of the corresponding locale. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

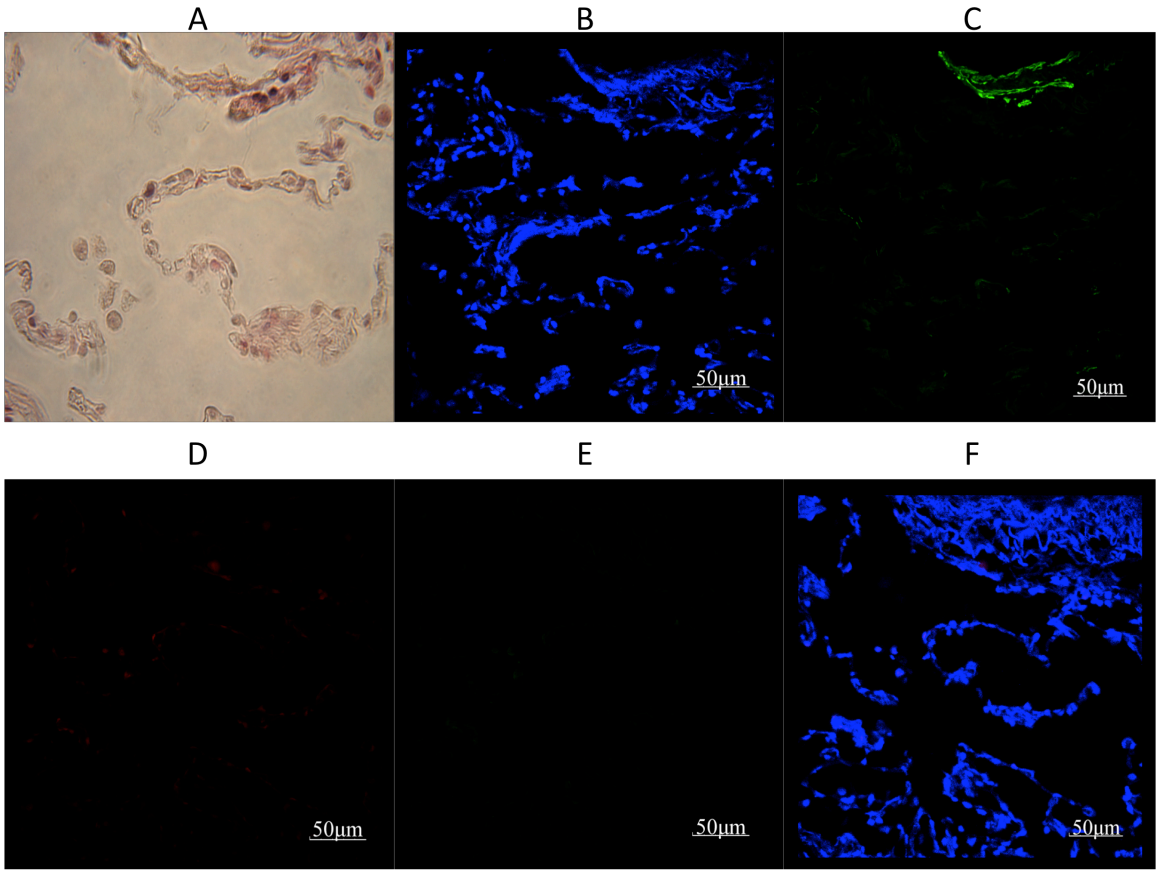

Figure A10: IHC analysis of the same locale in Figure A9 (normal lung) for expression of KLF4, PCNA and alpha-SMA proteins at 40x objective magnification. A: H&E of normal lung tissue. B: DAPI staining of the corresponding locale. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

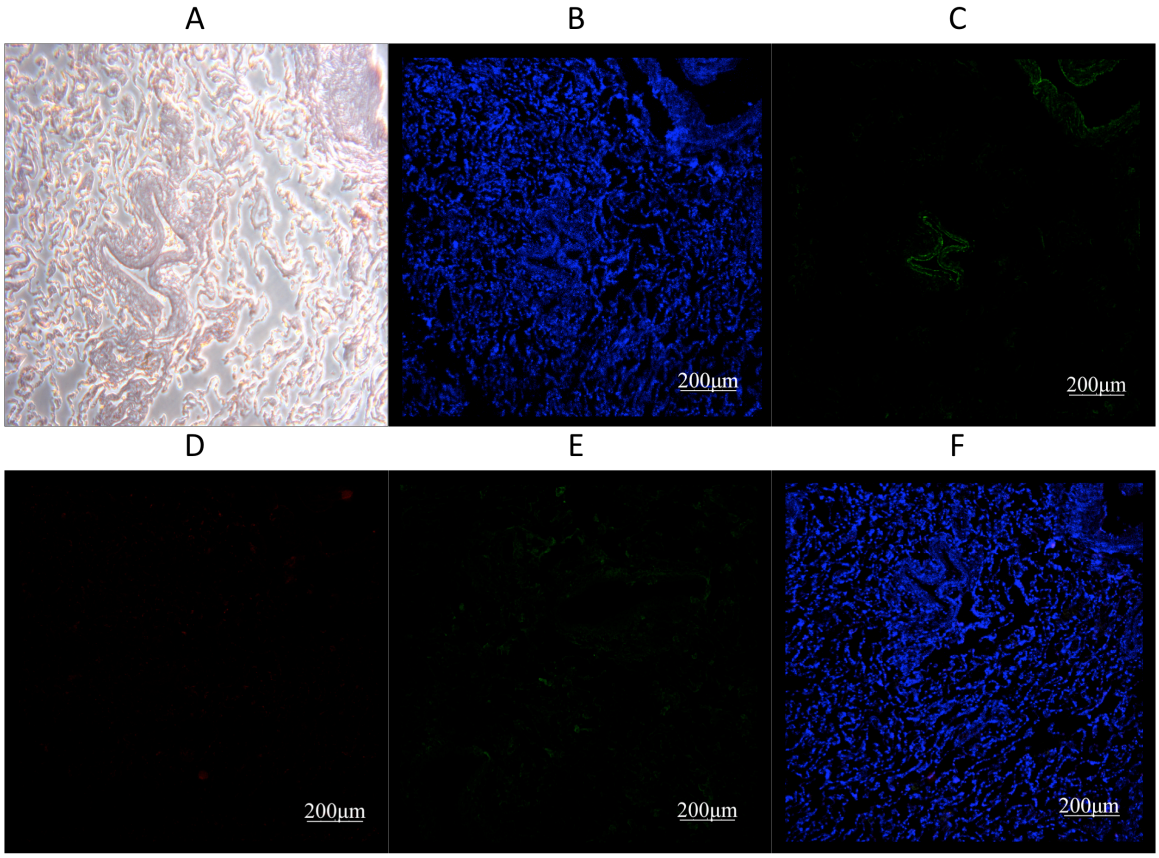

Figure A11: IHC analysis of a normal lung for expression of KLF4, PCNA and alpha-SMA proteins at 10x objective magnification. A: H&E of normal lung tissue. B: DAPI staining of the corresponding locale. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

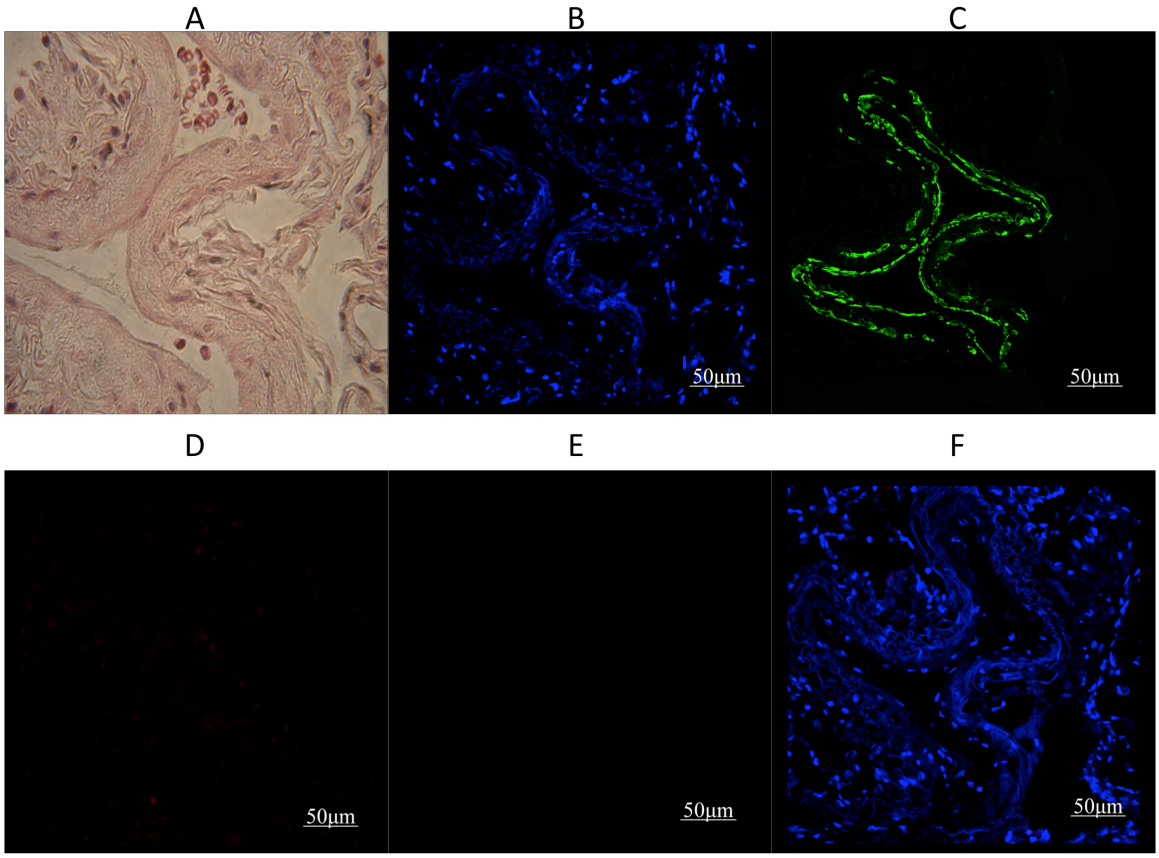

Figure A12: IHC analysis of the same locale in Figure A11 (normal lung) for expression of KLF4, PCNA and alpha-SMA proteins at 40x objective magnification. A: H&E of normal lung tissue. B: DAPI staining of the corresponding locale. C: Green fluorescent staining of is representative of alpha-SMA localization. D: Red fluorescent staining is representative of KLF4 localization E: Green fluorescent staining is representative of PCNA localization. F: Double IHC of PCNA (green) and KLF4 (red) with white representative of co-localization.

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CURRICULUM VITAE

John Robert Grimsley graduated from Hayfield Secondary School, Alexandra, Virginia, in 2003. He received his Bachelor of Science in Biology from George Mason University in 2008.