THE TISSUE ANGIOTENSIN SYSTEM IN THE LUNG: ROLES IN HUMAN PULMONARY FIBROSIS

By

My-Trang Thi Dang

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Microbiology and Molecular Genetics - Doctor of Philosophy

2014

ABSTRACT

THE TISSUE ANGIOTENSIN SYSTEM IN THE LUNG
ROLES IN HUMAN PULMONARY FIBROSIS

By

My-Trang Thi Dang

Idiopathic Pulmonary Fibrosis (IPF) is the most common form of interstitial lung disease with a 3-year median survival upon diagnosis. The lack of effective therapies in treating this disease highlights our incomplete understanding in the pathogenesis of IPF. The prevailing hypothesis is that IPF is a result of abnormal wound healing which consists of persistent injury and apoptosis to alveolar epithelial cells (AECs), aberrant fibroblast proliferation, and the accumulation of extracellular matrix proteins. Our laboratory has implicated a role of the angiotensin (ANG) system in these events.

In IPF, both angiotensinogen (AGT), the only known precursor to angiotensin II (ANGII), and Transforming Growth Factor-Beta (TGF-β_1) mRNA and protein are up-regulated, as well as the profibrotic peptide, ANGII. In human pulmonary fibroblasts, TGF-β_1-inducible *AGT* transcription is mediated by the core promoter spanning from -46 to +22. At the -20, -18, and -6 positions lies single nucleotide polymorphisms (SNPs) that have been shown to influence its transcription rate in hepatocytes. Our results in human pulmonary fibroblasts parallel those observed in hepatocytes where the CA haplotype at -20 and -6 respectively, had about a 1.5-fold increase in *AGT* transcription compared to the AG haplotype ($p = 0.011$). The increase in *AGT* transcription would result in an increase in ANGII, which we predict to be associated with

greater severity of IPF as measured by pulmonary function tests. Studies in IPF cohorts from the United States and Spain demonstrated that the CC genotype at -20 ($p = 0.0028$ for U.S. and $p = 0.017$ for Spain), the AA genotype at -6 ($p = 0.021$ for U.S.), and the CA haplotype ($p = 0.0048$ for U.S. and $p = 0.014$ for Spain) predicted lower diffusing capacity. Additionally, the Proline/Proline variant at codon 10 in *TGF-β_1* was also associated with lower diffusing capacity ($p = 0.0014$). Surprisingly, the results of both studies were only significant in males, reflecting the male bias of this disease.

Preliminary data indicates that in addition to inducing *AGT* transcription, TGF-β_1 also up-regulates cathepsin D and down-regulates ACE-2. Cathepsin D and AGT are both part of the rate-limiting step in the generation of ANGII whereas ACE-2 functions in its removal. This suggests that TGF-β_1 may cause an imbalance in the ANG system by favoring the ANGII producing axis. The mechanism by which ACE-2 is down-regulated has not been well studied. However, results from our lab suggests that this down-regulation may be related to ACE-2 ectodomain shedding or through a JNK-mediated mechanism as seen with inducers of ER-stress and cell-cycling in AECs.

For all their selfless sacrifices, I was given everything that they did not have. Without them, I would not be who I am today. I can never thank you enough. This accomplishment is dedicated to you, Mạ and Ba.

Kính tặng Ba Mẹ, Người đã hy sinh thầm lặng để cho con có những điều mà Ba Mẹ chưa từng có được. Không có Ba Mẹ, con không thể được như ngày hôm nay. Con không có lời nào diễn tả hết sự biết ơn dành cho Ba Mẹ. Xin gửi tặng Ba Mẹ thành quả mà con đạt được ngày hôm nay.

ACKNOWLEDGEMENTS

Words cannot express my deepest gratitude and appreciation for all the help, support, and guidance that I have received throughout this journey. My gratitude knows no bounds for the perfect mentor, Dr. Bruce Uhal - a friend who is understanding, patient, and encouraging, yet challenges my potential, even in times of self-doubt. I am eternally grateful to my Guidance Committee, Dr. Andrea Amalfitano, Dr. Karl Olson, Dr. Patrick Venta, and Dr. Vilma Yuzbasiyan-Gurkan for seeing my potential as a scientist, stimulating my intellectual growth, and for taking the time to be a part of this journey. My gratitude is extended to Dr. Sung Jin Kim for taking the time to be my Committee Chair. Without the great support from the Microbiology and Molecular Genetics Department and the College of Osteopathic Medicine at Michigan State University, I would not have had the opportunity to begin this challenging yet exciting adventure. Dr. Veronica Maher, Dr. Justin McCormick, and Mrs. Bethany Heinlen deserves more than my heartfelt gratitude for their never-ending encouragement, compassion, and dedication in helping me to succeed on this journey. To all my family and friends, I owe you an infinite amount of thanks for all your unwavering support, patience, and sincerity. Lastly, without financial support from PHS Grant HL-45136, the DO/PhD Program and the Graduate School at Michigan State University, the completion of this dissertation would have not been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

6MWT	6-Minute Walk Test
α-SMA	Alpha-Smooth Muscle Actin
AB/GS	Gilead
ACE	Angiotensin Converting Enzyme
ACEi	Angiotensin Converting Enzyme Inhibitor
ACE-2	Angiotensin Converting Enzyme-2
ACE-IPF	Anti-Coagulant Effectiveness in Idiopathic Pulmonary Fibrosis
AEC(s)	Alveolar Epithelial Cell(s)
AGCE-1	Angiotensinogen Core Promoter Element-1
AGCF-1	Angiotensinogen Core Promoter Element Binding Factor-1
AGT	Angiotensinogen
AIP	Acute Interstitial Pneumonia
ALAT	Latin American Thoracic Association
ANG1-7	Angiotensin 1-7
ANGI	Angiotensin I
ANGII	Angiotensin II
AP-1	Activation Protein-1
Arp-1	Nuclear Receptor Superfamily 2, Group F, Member 2

ARs	Angiotensin Receptors
ARB(s)	Angiotensin Receptor Blocker(s)
ARTEMIS-IPF	A Placebo-Controlled Trial of Ambrisentan in Idiopathic Pulmonary Fibrosis
AT	Angiotensin Receptor Type (1, 2)
ATG	Haplotype in *AGT* at -20, -18, and -6 respectively
ATS	American Thoracic Society
ATCC	American Type Culture Collection
BMS	Bristol-Myers-Squibb
CC	Celgene Corporation
CCL-2	Chemokine (C-C motif) Ligand 2
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CI	Confidence Interval
CKD	Chronic Kidney Disease
CNTO	Centocor
COP	Cryptogenic Organizing Pneumonia
COX-2	Cyclooxygenase-2
CT	Cycle Threshold
CTA	Haplotype in *AGT* at -20, -18, and -6 respectively
CTGF	Connective Tissue Growth Factor

CXCL Chemokine (C-X-C motif) Ligand (5, 10, 12)

CXCR-4 Chemokine (CXC) Receptor-4

D(D) Allele (or Genotype) for 287 bp deletion in intron 16 in ACE

df Degrees of Freedom

DIP Desquamative Interstitial Pneumonia

DL_{CO} Diffusing Capacity of the Lung for Carbon Monoxide

DRCT Double-Blinded Randomized Control Trial

DRs Direct Repeat Sequences

DSP Desmoplakin

DTT Dithiothreitol

EMSA(s) Electrophoretic Mobility Shift Assays

EMT Epithelial-to-Mesenchymal Transition

ER Endoplasmic Reticulum

ER-α Estrogen Receptor-Alpha

ERK Extra-cellular Signal Regulated Protein Kinase

ERS European Respiratory Society

ET-1 Endothelin-1

ESRD End Stage Renal Disease

FBS Fetal Bovine Serum

FEV_1 Forced Expiratory Volume in 1 Second

FEV_6	Forced Expiratory Volume in 6 Seconds
FG	FibroGen
FPF	Familial Pulmonary Fibrosis
FVC	Forced Vital Capacity
GC	Genzyme
HIF-1α	Hypoxia-Inducible Factor-1-Alpha
HNF-4	Hepatocyte Nuclear Factor-4
HRCT(s)	High-Resolution Computed Tomography Scan(s)
IFN-γ	Interferon-Gamma
IIP(s)	Idiopathic Interstitial Pneumonia(s)
IL	Interleukin (1-Alpha, 4, 6, 8, 10, 12, 13)
ILD(s)	Interstitial Lung Disease(s)
IPF	Idiopathic Pulmonary Fibrosis
JNK	Jun N-Terminal Kinase
JRS	Japan Respiratory Society
K_{CO}	Transfer Coefficient (DL_{CO}/V_{alv})
kDa	kilo-Daltons
LAP	Latency-Associated Peptide
LIP	Lymphoid Interstitial Pneumonia
LOXL-2	Lysyloxidase-like-2 Protein

LTBPs	Latent TGF-β Binding Proteins
LTRC	Lung Tissue Research Consortium
MAPK	Mitogen-Activated Protein Kinase
MEM	Minimal Essential Media
miR	Micro-RNA
mm Hg	millimeters of Mercury
MMP-1	Matrix Metalloproteinase-1
mTOR	Mechanistic Target of Rapamycin
MUC5B	Mucin 5B
MUSIC	Macitentan Use in an Idiopathic Pulmonary Fibrosis Clinical Study
NAFLD	Non-Alcoholic Fatty Liver Disease
NOD2	Nucleotide-Binding Oligomerization Domain Containing-2
NSIP	Non-Specific Interstitial Pneumonia
OR	Odds Ratio
PAI	Plasminogen Activator Inhibitor (type 1 or 2)
PANTHER-IPF	Prednisone, Azathioprine, and N-Acetylcysteine: A Study That Evaluates Response in Idiopathic Pulmonary Fibrosis
PBS	Phosphate Buffered Saline
PFTs	Pulmonary Function Tests
PHT	Pulmonary Hypertension

(P)RR	(Pro) Renin Receptor
QAX	Novartis
RAS	Renin-Angiotensin System
RB-ILD	Respiratory Bronchiolitis Interstitial Lung Disease
RCT	Randomized Control Trial
RGD	Arginine-Glycine-Aspartic Acid Motif
RTD	Renal Tubular Dysgenesis
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RTSF	Research Technology Support Facility
serpins	Serine Protease Inhibitors
SNP(s)	Single Nucleotide Polymorphism(s)
SP	Surfactant Protein (A, B, C, D)
SPPL-2C	Signal Peptide Peptidase Like-2C
STAT	Signal Transducer and Activator of Transcription (1, 3)
STEP-IPF	Sildenafil Trial of Exercise Performance in Idiopathic Pulmonary Fibrosis
STX	Stromedix (now owned by Biogen)
TβRI	Type I Transforming Growth Factor Beta Receptor
TβRII	Type II Transforming Growth Factor Beta Receptor
TAB	TAK-1 Binding Protein
TAK-1	TGF-β-activated Kinase

TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with 0.1% Tween 20
TERC	Telomerase RNA Component
TERT	Telomerase Reverse Transcriptase
TF(s)	Transcription Factor(s)
TGF-β_1	Transforming Growth Factor Beta (isoform type 1)
TLC	Total Lung Capacity
TLR-3	Toll-Like Receptor-3
TNF-α	Tumor Necrosis Factor-Alpha
TOLLIP	Toll Interacting Protein
UIP	Usual Interstitial Pneumonia
V_{alv}	Alveolar Volume
VEGF	Vascular Endothelial Growth Factor
WT	Wild-Type

CHAPTER 1

THE ANGIOTENSIN SYSTEM IN HUMAN DISEASES

Components of the Renin-Angiotensin System

General Overview.

The renin-angiotensin system (RAS) is described as a "peptidergic system with endocrine characteristics."[1] The starting substrate, angiotensinogen (AGT), generates the main effector peptide, angiotensin II (ANGII) through a series of enzymatic cleavage reactions. In the classical RAS that is well-known to regulate blood pressure, AGT is first cleaved by renin to form the decapeptide angiotensin I (ANGI). ANGI is cleaved by angiotensin converting enzyme (ACE) to the effector octopeptide, ANGII. ANGII can mediate its effects by binding to angiotensin receptors (ARs). The effects of ANGII can be predominantly opposed by its enzymatic product, angiotensin 1-7 (ANG1-7) which is generated by ACE-2. Apart from the classical RAS, local RAS exists in various organ systems such as the heart, brain, and lung.[2-4] In the lung, this system is independent of the endocrine RAS. Instead of relying on circulating renin and ACE to generate ANGII, it is dependent on other enzymes such as cathepsin D, tonin, cathepsin G, and chymase for the proteolytic conversion (**Figure 1.1**).[5] In order for homeostasis to occur, there must be a balance in the generation of ANGII (by the "ACE"-ANGII-AT_1 axis) and its degradation (by the ACE-2-ANG1-7-Mas axis). Dysfunction occurs when there is an imbalance in the ANG system resulting in disease phenotypes.

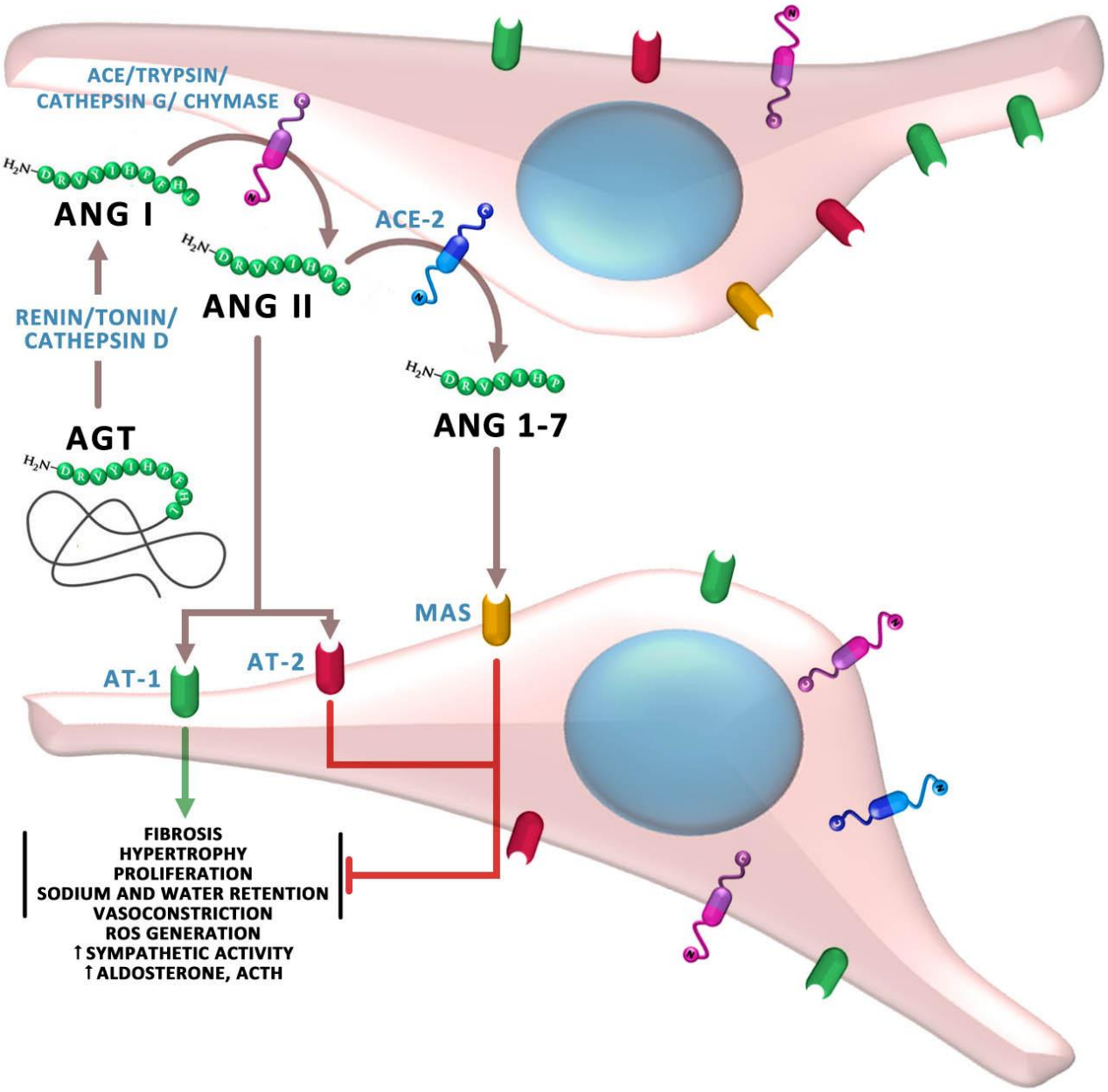

Figure 1.1. General overview of the enzymatic reactions in the angiotensin system. AGT = angiotensinogen; ANG = angiotensin; AT-1 = angiotensin type 1 receptor; AT-2 = angiotensin type 2 receptor. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

"ACE"-ANGII-AT_1 Axis: The ANGII Producing Arm.

AGT. Human *AGT* is a member of the serine protease inhibitor (serpins) superfamily containing 5 exons and 4 introns. The gene is located on chromosome 1q42-q43 and encodes a 61 kDa β_2-globulin protein, AGT - the starting substrate in the RAS. The initiation methionine, signal peptide, and most of the mature protein are encoded in the second exon.[6-7] The abundance of AGT is transcriptionally regulated (for more information, see **Chapter 3**).

Renin. Renin is an aspartyl protease encoded on chromosome 1q32. Renin catalyzes the conversion of AGT to ANGI. The active form of renin is generated from prorenin by an unknown pro-convertase that removes a 43-amino acid N-terminal segment in the active site cleft. Currently, it is thought that the uptake of prorenin and renin is mediated by the (Pro) Renin Receptor [(P)RR].[8]

ACE. ACE is a zinc-metallopeptidase with dipeptidyl carboxypeptidase activity. It is composed of 26 exons and 25 introns encoded on chromosome 17q23.[9] ACE cleaves the C-terminal Histidine-Leucine residue from ANGI to generate ANGII. Additionally, it can cleave the C-terminal Phenylalanine-Arginine residue from bradykinin to inactive it.[9]

Cathepsin D. Human cathepsin D is an aspartyl protease - a lysosomal hydrolase with an active site Aspartic Acid that is proteolytically active at acidic pH.[10] It contains 9 exons and 8 introns and is located on chromosome 11p15.[11] The mature cathepsin D form contains a heavy (34 kDa) and a light chain (14 kDa) that is proteolytically cleaved from the prepro-form (52 kDa)

in a multi-step reaction.[10] Cathepsin D functions in the alternative RAS by generating ANGI from AGT.

ANGI. ANGI is an intermediate product in the angiotensin (ANG) system and is the precursor to ANGII. ANGI is generated from AGT by the cleavage from aspartyl proteases such as renin or cathepsin D. In addition to this, a large fragment called des(ANGI)AGT is generated. Currently the only known function of des(ANGI)AGT is its anti-angiogenic effect in endothelial cells.[12]

Cathepsin G. Cathepsin G is a serine protease from the chymotrypsin superfamily and is one of the major constituents in the azurophilic granules of neutrophils.[13] It consists of 5 exons and 4 introns located on chromosome 14q11.2.[14] Interestingly, this region also encodes for a similar gene, *chymase.* The mature form of cathepsin G (28.5 kDa) is generated from the pre-pro form after cleavage by cathepsin C and additional proteases.[15] Cathepsin G is only active once it is released from the granules (optimum pH = 7-8).[16] The catalytic active site consists of Histidine, Aspartic Acid, and Serine residues.[17] Like ACE, it functions in the conversion of ANGI to ANGII.

Chymase. Chymase possess similar characteristics to cathepsin G; it is also a serine protease with chymotrypsin-like activity.[18] Initially, it is synthesized in a pre-pro form which undergoes a series of enzymatic cleavages to produce the mature form. Chymase is activated once it is released from the secretory granules of mast cells (optimum pH = 7-9).[19] Similar to ACE and cathepsin G, chymase can convert ANGI to ANGII.

ANGII. ANGII is the main effector peptide in the ANG system. It can increase sympathetic function, vasoconstriction, stimulate aldosterone release and sodium reabsorption, fibrosis, and induce proliferation or apoptosis depending on the cell-type.[20-21]

Angiotensin Receptors (ARs). ANGII mediated effects are through the ARs - primarily through AT_1 and AT_2. AT_1 activation results in vasoconstriction, reabsorption of water and sodium, cell proliferation, thrombosis, inflammation, fibrosis, and oxidative stress.[22] AT_2 activation results in effects that antagonizes AT_1-mediated effects resulting in vasodilation and inhibition of cell proliferation and inflammation.[22]

ACE-2-ANG1-7-Mas Axis: The ANGII Degrading Arm.

ACE-2. Human ACE-2 is a zinc metallopeptidase encoded on the X-chromosome.[23] It has homology to ACE with 42% sequence similarity.[24] ACE-2 cleaves the Phenylalanine from the C-terminal of ANGI or ANGII to respectively yield ANG1-9 or ANG1-7.[24] However, the affinity for ANGII is more than 400-fold greater than for ANGI, thereby favoring the generation of ANG1-7.[25]

ANG1-7. ANG1-7 is a product from the degradation of ANGII by ACE-2. It mediates its effect through the receptor Mas to promote vasodilation, anti-proliferation, and anti-hypertrophic effects - counteracting many of ANGII-mediated effects.[26-28] It is the main endogenous counterpart to ANGII.

Mas. Mas is a G-protein coupled-receptor that mediates the effects of ANG1-7 - most of which counteract the effects mediated by ANGII through AT_1. These effects include anti-proliferative and anti-fibrotic actions, vasodilation, diuresis and natriuresis.[29]

Mutations in the Angiotensin System and Human Diseases

General Overview.

A variety of mutations in components in the ANG system have been associated with a handful of disease phenotypes. Although the ANG system is most commonly associated with the regulation of blood pressure and the major focus of mutations in the ANG system are in this area, mutations in this system also extend beyond this disease phenotype. This section will address the association between mutations in the ANG system with the risk, severity, and/or progression of various diseases.

Renal Tubular Dysgenesis.

Currently, Renal Tubular Dysgenesis (RTD) is the only Mendelian disease associated with multiple mutations in the RAS (**Figure 1.2**).[30] RTD is often diagnosed in fetuses and newborns of mothers with a history multiple miscarriages. This severe disease is inherited as an autosomal recessive disorder characterized by the paucity of differentiated proximal tubules on histology.[30] Affected newborns are often stillborn or die in utero, experience severe oligohydramnios, hypotension and exhibit Potter's sequence.[30] In 2005, mutation screenings in

nine families with a total of 16 affected offsprings revealed 5 mutations in renin, 2 mutations in ACE, and single mutations in AGT and AT_1 - all resulting in the absence or ineffective production of ANGII.[30] As of today, similar mutations have been reported in 50 unrelated families.[31] As opposed to this disease where the pathogenesis is a result of ineffective production of ANGII, the following diseases that will be discussed are associated with an over-production of ANGII.

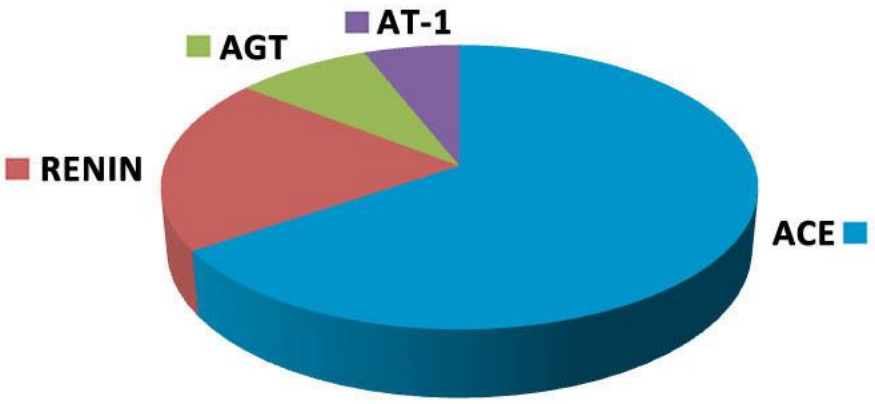

Figure 1.2. Distribution of mutations in the RAS associated with RTD. 58.3% of affected individuals are homozygous for these mutations while 41.7% are compound heterozygotes.[30]

Essential Hypertension.

In adults, hypertension is defined with a systolic blood pressure > 140 mm Hg or a diastolic blood pressure > 90 mm Hg. Linkage analysis in two sibship cohorts from Utah and Paris suggested a role of *AGT* polymorphisms with essential hypertension.[32] Additional studies have demonstrated significant associations of several *AGT* polymorphisms with essential hypertension - of which, M235T is one of the most well studied. The frequency of the M235T single nucleotide polymorphism (SNP) is greatly influenced by ethnicity - with higher frequency

of the T235 in Africans (0.90-0.95) and Asians (0.75) compared to Caucasians (0.40).[33-34] A meta-analysis involving 5,493 Caucasian patients showed significant association with the T235 *AGT* SNP and hypertension, especially in patients with a positive family history of hypertension and those with more severe forms.[35] A multiple regression analysis in 347 Japanese patients showed that the T235 allele was a significant predictor in patients less than 50 years old. In an African Caribbean cohort, a weak association between hypertension and the M235T SNP was found.[36] The presence of the T235 allele was also associated with significant increases in plasma AGT concentrations in several cohorts.[32, 34] The T235 is in almost complete linkage disequilibrium with the *AGT* promoter variant, A-6, whose haplotype is also associated with essential hypertension.[32, 37] Inoue et al. demonstrated that the presence of the A allele at -6 resulted in significant increases in *AGT* transcription in the human hepatoma cell line, HepG2.[37] In addition to the G-6A promoter variant, there are also two other promoter variants located at the -18 and -20 positions that are also in linkage disequilibrium with T235. Due to rarity of the C-18T SNP in the human population, it is difficult to determine its association with hypertension. In a Japanese cohort, Sato et al. found a significant increase in the frequency of the T-18 allele in hypertensive individuals (3.5%) compared to controls (1%).[38] On the other hand, the A-20C SNP showed significant association with plasma AGT concentrations[39-40] and essential hypertension.[39]

Pre-eclampsia.

Pre-eclampsia is a hypertensive disorder of pregnancy defined as the development of persistently high blood pressure ($\geq$ 140 mm Hg systolic or $\geq$ 90 mm Hg diastolic blood pressure) on 2 occasions at least 4 hours apart after 20 weeks gestation with the presence of proteinuria or new onset of thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, or cerebral or visual symptoms.[41] Currently, it is one of the leading causes of maternal and perinatal morbidity and mortality. As in essential hypertension, the T235 *AGT* SNP is also significantly associated with pre-eclampsia.[42-44] An association with the M174 *AGT* SNP has also been found.[45]

A haplotype analysis demonstrated that the A1035-M174-T235 *AGT* haplotype was associated with a 2.1 fold increased risk of pre-eclampsia (95% CI: 1.4-3.4).[45] Interestingly, several maternal/newborn genotypes were identified by Procopciuc et al. as contributors to the risk of pre-eclampsia in a Romanian cohort.[46] In this study, significant risk of pre-eclampsia was increased in mothers who were homozygous for T235 in *AGT,* C1166 in AT_1, A3123 in AT_2, G83 in *renin*, and the 287 bp deletion (D) in intron 16 in ACE. Significant risk of pre-eclampsia were associated when both mother and newborn had the presence of the following alleles: T235 *AGT* (OR = 6.67), deletion or G2350 in *ACE* (OR = 5.00 and 3.33 respectively), C1166 in AT_1 (OR = 2.72), or G83 in *renin* (OR = 7.8).[46] The *ACE* deletion accounts for at most 50% of the inter-individual variation in the serum concentration of ACE and is significantly associated with higher

concentrations and activity as well.[47] The association of the *ACE* deletion is less clear in essential hypertension.

Kidney Disease.

Carriers of the *ACE* deletion have a higher risk of developing chronic kidney disease (CKD) or end-stage renal disease (ESRD) compared to those with the *ACE* insertion.[48-50] A meta-analysis revealed an additive effect of hypertension and the *ACE* deletion with the risk of CKD.[51] Additionally, in Asians, the male sex has an additive effect on the risk of CKD, which parallels previous findings in Japanese and Korean cohorts.[51-53] It is thought that the variability in the additive nature of the male sex and the *ACE* deletion is related to androgen sensitivity due to the higher utilization of male sex hormones between males and females in Asians than Caucasians.[51] A meta-analysis by Zhou et al. demonstrated an association between the *ACE* deletion polymorphism and ESRD risk in IgA nephropathy patients (D allele with $p = 0.01$ and DD genotype with $p = 0.003$).[54] In Korean male patients, the *AGT* M235T polymorphism was associated with the progression of IgA nephropathy ($p = 0.019$).[55]

Liver Disease.

Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of fat in hepatocytes resulting in hepatic steatosis or cirrhosis.[56] As its name implies, this accumulation of fat is unrelated to alcohol consumption. In a Japanese cohort, the presence of the A allele in

the *AT$_1$* rs3772622 SNP was associated with an OR = 1.95 for developing NAFLD ($p = 1.2 \times 10^{-6}$).[57] Additionally, five other SNPs in *AT$_1$* were also significantly associated, rs3772633, rs2276736, rs3772630, rs3772627, and rs3772622.[57] Haplotype studies involving these five SNPs revealed that the GCGTA haplotype (at rs3772633, rs2276736, rs3772630, rs3772627, and rs3772622, respectively) increased the risk of NAFLD while the ATATG haplotype is protective ($p = 5.7 \times 10^{-6}$ and 7.7×10^{-7}).[57] However, in Asian Indians, the ACGCA haplotype is protective while the presence of the G allele in rs3772622 was associated with an increase in the risk of fibrosis ($p = 0.003$).[58] The discrepancies in these findings suggests an influence of ethnicity on the effects of *AT$_1$* variants and NAFLD.

Excess deposition of extracellular matrix in the perisinusoidal and periportal spaces of the liver constitute liver fibrosis. The final disease stage is liver cirrhosis. Xiao et al. demonstrated a significant association between the A-20C ($p = 0.007$) and G-6A ($p = 0.042$) variants in *AGT* with liver cirrhosis in patients with chronic hepatitis B.[59] The C allele at the -20 position (OR = 2.83) and the G allele at the -6 position (OR = 1.80) are important in the progression of liver cirrhosis.[59] Additionally, the allele frequencies at the -20 ($p = 0.004$) and -6 ($p = 0.025$) positions between affected and control populations were significantly different.[59]

In addition to the above diseases that are associated with mutations in the ANG system, various other disease phenotypes have also been implicated (**Table 1.1**).

Table 1.1. Association of known diseases with a genetic component related to the angiotensin system (compiled from Online Mendelian Inheritance in Man).[60]

GENE	PHENOTYPE
RENIN	Familial Juvenile Hyperuricemic Nephropathy
	Renal Tubular Dysgenesis
ACE	Renal Tubular Dysgenesis
	Susceptibility to Alzheimer Disease
	Susceptibility to Myocardial Infarction
	Progression of severe Acute Respiratory Distress Syndrome
	Hemorrhagic Stroke
AGT	Renal Tubular Dysgenesis
	Susceptibility to Essential Hypertension
	Susceptibility to Pre-eclampsia
AT_1	Essential Hypertension
	Renal Tubular Dysgenesis
CATHEPSIN D	Neuronal Ceroid Lipofuscinosis

ACE = angiotensin converting enzyme; AGT = angiotensinogen;

AT_1 = angiotensin receptor type 1

Conclusions

A balance in the ANG system is critical in maintaining homeostasis. Mutations in the ANG system can upset this balance resulting in dysfunction and observable disease phenotypes. Although the ANG system is most commonly associated with regulating blood pressure, mutations in this system are also observed in other disease phenotypes such as RTD, liver fibrosis/cirrhosis, and chronic kidney disease. In the diseases associated with a fibrotic phenotype, the balance favors the production of the effector peptide in the ANG system, ANGII. ANGII is known to be profibrotic in various organs systems including the heart, liver, kidney, and also the lungs. It is hypothesized that the presence of functional mutations in components of the ANG system favors the generation of ANGII, thereby promoting the fibrotic response. This concept was used as the foundation for studying the role of the ANG system in human pulmonary fibrosis that will be discussed in the following chapters.

REFERENCES

REFERENCES

1. Martin, P., Mehr, A.P., and Kreutz, R. Physiology of local renin-angiotensin systems. *Physiol. Rev.* 86:747-803 (2006).

2. Lindpainter, K., Jin, M., Wilhelm, M.J., Suzuki, F., Linz, W., Schoelkens, B.A., and Ganten, D. Intracardiac generation of angiotensin and its physiologic role. *Circulation.* 77:I18-I23 (1988).

3. Campbell, D.J., Bouhnik, J., Menard, J., and Corvol, P. Identity of angiotensinogen precursors of rat brain and liver. *Nature.* 308:206-208 (1984).

4. Filippatos, G., Tilak, M., Pinillos, H., and Uhal, B.D. Regulation of apoptosis by angiotensin II in the heart and lungs (review). *Int. J. Mol. Med.* 7:272-280 (2001).

5. Uhal, B.D., Dang, M.T., Li, X., and Abdul-Hafez, A. Angiotensinogen gene transcription in pulmonary fibrosis. *Int. J. Pept.* 2012;2012:875910. doi: 10.1155/2012/875910 (2012).

6. Braiser, A.R., Han, Y., and Sherman, C.T. Transcriptional regulation of angiotensinogen gene expression. *Vitam. Horm.* 57:217-247 (1997).

7. Jeunemaitre, X. Genetics of the human renin-angiotensin system in *Handbook of Experimental Pharmacology; Angiotensin.* Edited by T. Unger and B.A. Schölkens. 103:173-206. Springer, New York, NY, USA (2004).

8. Nguyen, G., and Contrepas, A. Chapter 3: Renin, Prorenin, and the (Pro) Renin Receptor in *Renin Angiotensin System and Cardiovascular Disease*. Edited by DeMello, W. and Frohlich, E. 3.15-3.24 (Humana Press, New York, NY, USA 2010).

9. Mattei, M.G., Hubert, C., Alhenc-Gelas, F., Roeckel, N., Corvol, P., and Soubrier, F. Angiotensin I converting enzyme gene is on chromosome 17. *Cytogenet Cell Genet.* 51:1041-1045 (1989).

10. Masson, O., Bach, A.S., Derocq, D., Prébois, C., Laurent-Matha, V., Pattingre, S., and Liaudet-Coopman, E. Pathophysiological functions of cathepsin D: targeting its catalytic activity versus its protein binding activity? *Biochimie.* 92:1635-1643 (2010).

11. Augereau, P., Garcia, M., Mattei, M.G., Cavailles, V., Depadova, F., Derocq, D., Capony, F., Ferrara, P., and Rochefort, H. Cloning and sequencing of the 52K cathepsin D complementary deoxyribonucleic acid of MCF7 breast cancer cells and mapping on chromosome 11. *Mol. Endocrinol.* 2:186-192 (1988).

12. Célérier, J., Cruz, A., Lamandé, N., Gasc, J.M., and Corvol, P. Angiotensinogen and its cleaved derivatives inhibit angiogenesis. *Hypertension.* 39:224-228 (2002).

13. Korkmaz, B., Horwtiz, M.S., Jeene, D.E., and Gauthier, F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharm. Rev.* 62:726-759 (2010).

14. Zimmer, M., Medcalf, R.L., Fink, T.M., Mattmann, C., Lichter, P., and Jenee, D.E. Three human elastase-like genes coordinately expressed in the myelomonocyte lineage are organized as a single genetic locus on 19pter. *Proc. Natl. Acad. Sci. USA.* 89:8215-8219 (1992).

15. Adkison, A.M., Raptis, S.Z., Kelley, D.G., and Pham, C.T., Dipeptidyl peptidase I activates neutrophil-derived serine proteases and regulates the development of acute experimental arthritis. *J. Clin. Invest.* 109:363-371 (2002).

16. Burster, T., Macmillan, H., Hou, T., Boehm, B.O., and Mellins, E.D. Cathepsin G: roles in antigen presentation and beyond. *Mol. Immunol.* 47:658-665 (2010).

17. Hohn, P.A., Popescu, N.C., Hanson, R.D., Salvesen, G., and Ley, T.J. Genomic organization and chromosomal localization of the human cathepsin G gene. *J. Biol. Chem.* 264:13412-13419 (1989).

18. Heuston, S., and Hyland, N.P. Chymase inhibition as a pharmacological target: a role in inflammatory and functional gastrointestinal disorders? *Br. J. Pharmacol.* 167:732-740 (2012).

19. Takai, S., Jin, D., and Miyazaki, M. New approaches to blockade of the renin-angiotensin-aldosterone system: chymase as an important target to prevent organ damage. *J. Pharmacol. Sci.* 113:301-309 (2010).

20. Ardaillou, R. Active fragments of angiotensin II: enzymatic pathways of synthesis and biological effects. *Curr. Opin. Nephrol. Hypertens.* 6:28-34 (1997).

21. Santos, R.A.S., Ferreira, A.J., and Silva, A.C.S. Recent advances in the angiotensin-coverting enzyme 2-angiotensin-(1-7)-Mas axis. *Exp. Physiol.* 93:519-527 (2008).

22. Ferrario, C.M., Trask, A.J., and Jessup, J.A. Advances in the biochemical and functional roles of angiotensin converting enzyme 2 and angiotensin-(1-7) in the regulation of cardiovascular function. *Am. J. Physiol. Heart Circ.* 289:H2281-H2290 (2005).

23. Crackower, M., Sarao, A., Oudit, G.Y., Yagil, C., Kozieradzki, I., Scanga, S.E., Oliveira-dos-Santos, A.J., da Costa, J., Zhang, L., Pei, Y., Scholey, J., Ferrario, C.M., Manoukian, A.S., Chappell, M.C., Backx, P.H., Yagil, Y., and Penninger, J.M. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature.* 417:822-828 (2002).

24. Tipnis, S.R., Hooper, N.M., Hyde, R., Karran, E., Christie, G., and Turner, A.J. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J. Biol. Chem.* 275:33238-33243.

25. Vickers, C., Hales, P., Kaushik, V., Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J. Biol. Chem.* 277:14838-14843 (2002).

26. Santos, R.A.S., Silva, A.C.S., Maric, C., Silva, D.M., Machado, R.P., de Buhr, I., Heringer-Walther, S., Pinheiro, S.V., Lopes, M.T., Bader, M., Mendes, E.P., Lemos, V.S., Campagnole-Santos, M.J., Schultheiss, H.P., Speth, R., and Walther, T. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc. Natl. Acad. Sci. USA.* 100:8258-8263.

27. Santos, R.A.S., Campagnole-Santos, M.J., and Andrade, S.P. Angiotensin-(1-7): an update. *Regul. Pept.* 91:45-62 (2000).

28. Ferrario, C.M., Counterregulatory actions of angiotensin-(1-7). *Hypertension.* 30:535-541 (1997).

29. Iwai, M., and Horiuchi, M. Devil and angel in the renin-angiotensin system: ACE-angiotensin II-AT1 receptor axis vs. ACE2-angiotensin-(1-7)-Mas receptor axis. *Hypertension Res.* 32:533-536 (2009).

30. Gribouval, O., Gonzales, M., Neuhaus, T., Aziza, J., Bieth, E., Laurent, N., Bouton, J.M., Feuillet, F., Makni, S., Amar, H.B., Laube, G., Delezoide, A., Bouvier, R., Dijoud, F., Ollagnon-Roman, E., Roume, J., Joubert, M., Antignac, C., and Gubler, M.C. Mutations in genes in the renin-angiotensin system are associated with autosomal recessive renal tubular dysgenesis. *Nature Gen.* 37:964-968 (2005).

31. Gubler, M.C. Renal tubular dysgenesis. *Pediatr. Nephrol.* 29:51-59 (2014).

32. Jeunemaitre, X. Soubrier, F., and Kotelevtsev, Y.V. Molecular basis of human hypertension: role of angiotensinogen. *Cell.* 71:169-180 (1992).

33. Rotimi, C., Morrison, L., Cooper, R., Oyejide, C., Effiong, E., Ladipo, M., Osotemihen, B., and Ward, R. Angiotensinogen gene in human hypertension. Lack of an association of the 235T allele among African Americans. *Hypertension.* 24:591-594 (1994).

34. Bloem, L.J., Manatunga, A.K., Tweksbury, D.A., and Pratt, J.H. The serum angiotensinogen concentration and variants of the angiotensinogen gene in white and black children. *J. Clin. Invest.* 95:948-953 (1995).

35. Kunz, R., Kreutz, R., Beiger, J., Distler, A., and Sharma, A.M. Association between the angiotensinogen 235-T variant and essential hypertension in whites: a systemic review and methodological appraisal. *Hypertension.* 30:1331-1337 (1997).

36. Forrester, T., McFarlane-Anderson, N., Bennet, F., WIlks, R., Puras, A., Cooper, R., Rotimi, C., Durazo, R., Tewksbury, D., and Morrison, L. Angiotensinogen and blood pressure among blacks: findings from a community survey in Jamaica. *J. Hypertens.* 14:315-321 (1996).

37. Inoue, I. Nakajima, T., Williams, C.S., Quackenbush, J., Puryear, R., Power, M., Cheng, T., Ludwig, E.H., Sharma, A.M., Hata, A., Jeunemaitre, X., and Lalouel, J.M. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. *J. Clin. Invest.* 99:1786-1797 (1997).

38. Sato, N., Katsuya, T., Rakugi, H., Takami, S., Nakata, Y., Miki, T., Higaki, J., and Ogihara, T. Association of variants in critical core promoter element of angiotensinogen gene with increased risk of essential hypertension in Japanese. *Hypertension.* 30:321-325 (1997).

39. Ishigami, T., Umemura, S., Tamura, K., Hibi, K., Nyui, N., Kihara, M., Yabana, M., Watanabe, Y., Sumida, Y., Nagahara, T., Ochiai, H., and Ishii,, M. Essential hypertension and 5' upstream core promoter region of human angiotensinogen gene. *Hypertension.* 30:1325-1330 (1997).

40. Balam-Ortiz, E., Esquivel-Villarreal, A., Huerta-Hernandez, D., Fernandez-Lopez, J.C., Alfaro-Ruiz, L., Muñoz-Monroy, O., Gutierrez, R., Figueroa-Genis, E., Carrillo, K., Elizalde, A., Hidalgo, A., Rodriguez, M., Urushihara, M., Kobori, H., and Jimenez-Sanchez, G. Hypercontrols in genotype-phenotype analysis reveal ancestral haplotypes associated with essential hypertension. *Hypertension.* 59:847-853 (2012).

41. American College of Obstetricians and Gynecologists. Hypertension in Pregnancy. http://www.acog.org/Resources_And_Publications/Task_Force_and_Work_Group_Reports/Hypertension_in_Pregnancy. Last visited February 2014 (2013).

42. Ward K., Hata, A., Jeunemaitre, X., Helin, C., Nelson, L., Namikawa, C., Farrington, P.F., Ogasawara, M., Suzumori, K., and Tomoda, S. A molecular variant of angiotensinogen associated with preeclampsia. *Nat. Genet.* 4:59-61 (1993).

43. Kobashi, G. Genetic and environmental factors associated with the development of hypertension in pregnancy. *J. Epidemiol*. 16:1-8 (2006).

44. Zafarmand, M.H., Franx, A., Sabour, S., Van Der Schouw, I.T., Grobbee De Leeuw, P.W., and Bots, M.L. The M235T variant of the angiotensinogen gene is related to development of self-reported hypertension during pregnancy: the Prospect-EPIC Cohort Study. *Hypertens. Res*. 31:1299-1305 (2008).

45. Levesque, S., Moutquin, J.M., Lindsay, C., Roy, M.C., and Rousseau, F. Implication of an AGT haplotype in a multigene association study with pregnancy hypertension. *Hypertension*. 43:71-78 (2004).

46. Procopciu, L, Garacostea, G., Zaharie, G., Puscas, M. Iordache, G., Popa, M., Colcear, D., Olteanu, I., and Stamatian, F. Maternal/newborn genotype contribution of the rennin-angiotensin system (Met235Thr, Thr74Met, I/D-ACE, A2350G-ACE,A1155C-AT1R, 83A/G-REN) to the risk of pre-eclampsia: a Romanian study. *J. Renin Angiotensin Aldosterone Syst.* 12:539-548 (2011).

47. Rigat, B., Hubert, C., Alhenc−Gelas, F., Cambien, F., Corvol, P., and Soubrier, F.. An insertion/deletion polymorphism in the angiotensin I‐converting enzyme gene accounting for half the variance of serum enzyme levels. *J. Clin. Invest*. 86:1343-1346 (1990).

48. Qin, Y.H., Zhou, T.B., Su, L.N., Lei, F.Y., Huang, W.F., and Zhao, Y.J. Association between ACE polymorphism and risk of IgA nephropathy: a meta-analysis. *J. Renin Angiotensin Aldosterone Syst.* 12:215-223 (2011).

49. Wang, F., Fang, Q., Yu, N., Zhao, D., Zhang, Y., Wang, J., Wang, Q., Zhou, X., Cao, X., and Fan, X. Association between genetic polymorphism of the angiotensin-converting enzyme and diabetic nephropathy: a meta-analysis comprising 26,580 subjects. *J. Renin Angiotensin Aldosterone Syst*. 13:161-174 (2012).

50. Zhou, T.B., Yin, S.S., and Qin, Y.H. Association between angiotensin-converting enzyme insertion/deletion gene polymorphism and end-stage renal disease susceptibility. *J. Renin Angiotensin Aldosterone Syst.* 15:22-31 (2012).

51. Lin, C., Yang, H., Wu, C., Lee, H., Lin, Y., Lu, K., Chu, C., Lin, F. Kao, S., and Su, S. Angiotensin-converting enzyme insertion/deletion polymorphism contributes high risk for chronic kidney disease in Asian male with hypertension - a meta-regression analysis of 98 observational studies. *PLoS.* 9:1-16 (e87604) (2014).

52. Nakajima, S., Baba, T., and Yajima, Y. Is ACE gene polymorphism a useful marker for diabetic albuminuria in Japanese NIDDM patients? *Diabetes Care.* 19:1420-1422 (1996).

53. Park, H.C., Choi, S.R., Kim, B.S., Lee, T.H., Kang, B.S., Choi, K.H., Lee, H.Y., Han, D.S., and Ha, S.K. Polymorphism of the ACE Gene in dialysis patients: overexpression of DD genotype in type 2 diabetic end-stage renal failure patients. *Yonsei Med. J*. 46:779-787 (2005).

54. Zhou, T.B., Yin, S.S., and Liang, R. A meta-analysis of the association between angiotensin-converting enzyme insertion/deletion gene polymorphism and end-stage renal disease risk in IgA nephropathy patients. *J. Renin Angiotensin Aldosterone Syst.* 14: 235-241 (2013).

55. Kim, S.M., Chin, H.J., Oh, Y.K., Kim, Y.S., Kim, S., and Lim, C.S. Blood pressure-related genes and the progression of IgA nephropathy. *Nephron. Clin. Pract.* 113:c301-c308 (2009).

56. Chalasani, N., Younossi, Z., Lavine, J.E., Diehl, A.M., Brunt, E.M., Cusi, K., Charlton, M., and Sanyal, A.J. The Diagnosis and Management of Non-Alcoholic Fatty Liver Disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatol.* doi: 10.1002/hep.25762 (2012).

57. Yoneda, M., Hotta, K., Nozaki, Y., Endo, H., Uchiyama, T., Mawatar, H., Iida, H., Kato, S., Fujita, K., Takahaashi, H., Kirikoshi, H., Kobayashi, N., Inamori, M., Abe, Y., Kubota, K., Saito, S., Maeyama, S., Wada, K., and Nakajima, A. Association between angiotensin II type 1 receptor polymorphisms and the occurrence of nonalcoholic fatty liver disease. *Liver Inter.* 29:1078-1085.

58. Zain, S.M., Mohamed, Z., Mahadeva, S., Rampal, S., Basu, R.C., Cheah, R.L., Salim, A., and Mohamed, R. Susceptibility and gene interaction study of the angiotensin II type 1 receptor (AGTR1) gene polymorphisms with non-alcoholic fatty liver disease in a multi-ethnic population. *PLoS.* 8:e58538. doi:10.1371/journal.pone.0058538 (2013).

59. Xiao, F., Wei, H., Song, S., Li, G., and Song, C. Polymorphisms in the promoter region of the angiotensinogen gene are associated with liver cirrhosis in patients with chronic hepatitis B. *J. Gastroenterol. Hepatol.* 21:1488-1491 (2006).

60. OMIM Online Mendelian Inheritance in Man: An Online Catalog of Human Genes and Genetic Disorders. John Hopkins University. omim.org Last visited May 2014 (2014).

CHAPTER 2

IDIOPATHIC PULMONARY FIBROSIS

Classification of Idiopathic Pulmonary Fibrosis

Interstitial Lung Diseases.

Interstitial Lung Diseases (ILDs), also referred to as diffuse parenchymal lung diseases, are disorders with underlying inflammation and/or fibrosis of the pulmonary interstitium. Albeit, that the effect is most prominent in the interstitium, the alveoli and small airways can also be affected.[1] Over 200 entities are classified under the umbrella of ILDs.[2] However, the majority of ILDs are from Idiopathic Pulmonary Fibrosis (IPF), connective-tissue disease-associated ILDs, sarcoidosis, and hypersensitivity pneumonitis[3] - with IPF being the most common type of ILD. This large group of heterogeneous disorders can be classified into four major groups: ILDs of known cause, idiopathic interstitial pneumonias, granulomatous ILDs, or rare/other ILDs (**Figure 2.1**).

Idiopathic Interstitial Pneumonias.

Idiopathic interstitial pneumonias (IIPs) represent a group of diffuse parenchymal lung diseases with unknown etiologies. Their diagnoses require exclusions of known causes of ILDs, such as exposure to medications or drugs (i.e. amiodarone, bleomycin, methotrexate, or chloramphenicol) and the co-existence of auto-immune diseases (i.e. systemic lupus erythematosus, sarcoidosis, rheumatoid arthritis, or Sjorgen's syndrome).[1-4] IIPs can be classified into three categories: major, rare, and unclassifiable (**Figure 2.1**).[4] The diagnoses of IIPs are distinguished from one another based on histologies from lung biopsies, radiographical

findings on high-resolution computed tomography scans (HRCT) or x-rays, history, and physical examination (**Table 2.1**).[1,4] The histological pattern provides the key clue in differentiating IIPs. Most of the histological patterns are reflected in the names of the disease entity which were first introduced by Liebow and Carrington.[2]

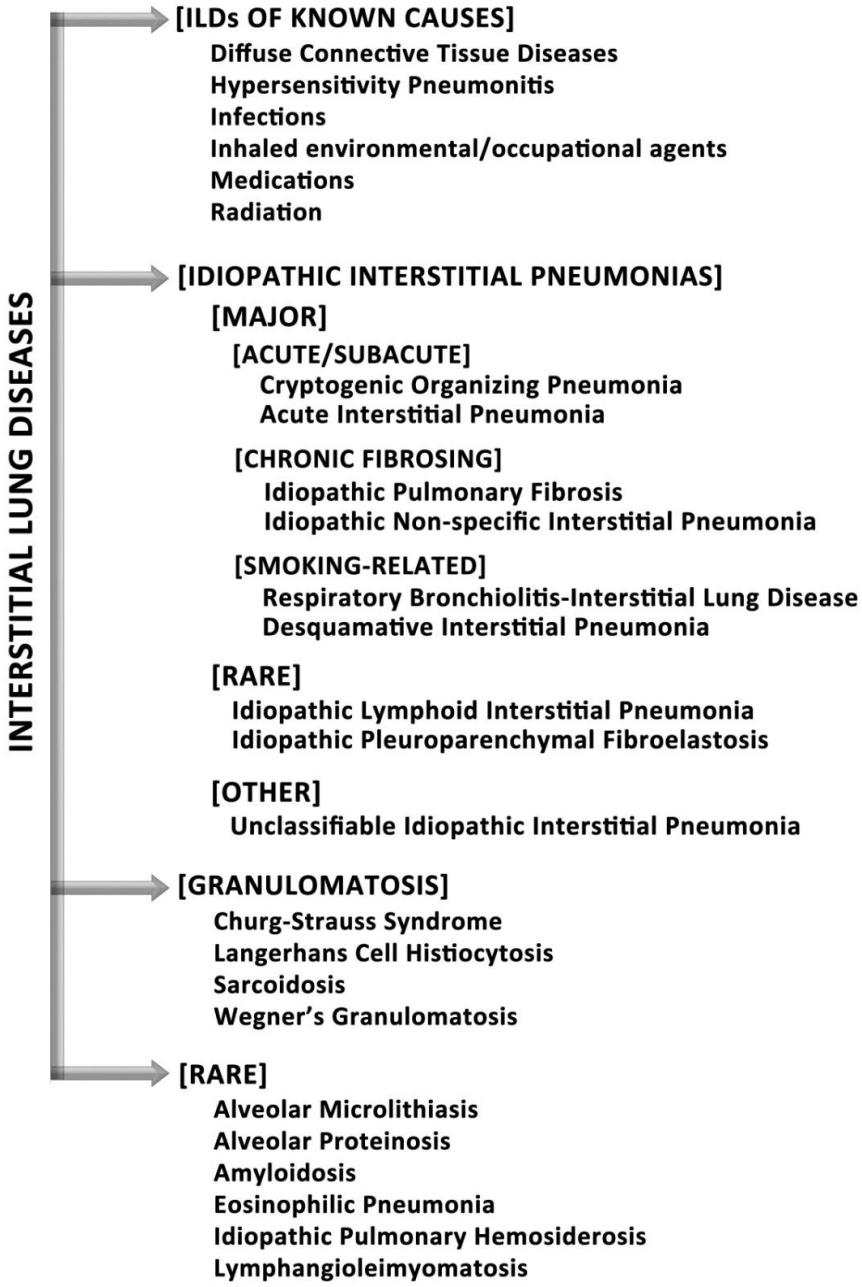

Figure 2.1. Classification of interstitial lung diseases (adapted with revisions from Peroš-Golubičić et al. and ATS/ERS).[1]

Table 2.1. **Key findings for distinguishing IIPs (adapted and revised from Peros--Golubicic and Travis et al.).**

DISEASE	HISTOLOGY PATTERN	HRCT FINDINGS	FREQUENCY
IPF	Usual interstitial pneumonia	Honey-combing, traction bronchietasis	55%
NSIP	Non-specific interstitial pneumonia	Ground-glass, traction bronchietasis	25%
RB-ILD	Respiratory bronchiolitis	Centrilobular changes	15%
DIP	Desquamative interstitial pneumonia	Ground-glass	
COP	Organizing pneumonia	Ground-glass	3%
AIP	Diffuse alveolar damage	Ground-glass, traction bronchietasis	< 1%
LIP	Lymphoid interstitial pneumonia	Ground-glass	<1%

General Overview of Idiopathic Pulmonary Fibrosis

Epidemiology.

IPF, also known as cryptogenic fibrosing alveolitis, is the most common form of interstitial lung disease with an estimated prevalence of about 20 per 100,000.[5] It is a "chronic, progressive, and irreversible" condition with a bias towards males in the fifth to eighth decade of life.[5] Upon diagnosis, the mean survival is three years. Currently, the only therapy to prolong survival is lung transplantation; however, this option is limited to a minority of affected patients.[5] Occupational exposure to livestock and dust from textiles, metals, woods, stone, sand, and silica are implicated as being risk factors for development of IPF (OR = 1.58 - 2.44).[6] Additionally, cigarette smoking is a strong risk factor (especially with a $\geq$ 20 pack-years smoking history).[7] Due to its rarity, IPF has been misdiagnosed and under-recognized.

Clinical Presentation.

Most patients present with a chief complaint of dyspnea and a dry cough that is refractory to antitussive agents.[8] Symptoms are usually present for at least 6 months and the diagnosis of IPF is often made when the disease has progressed due to the gradual and variable nature of its course. Lung auscultation will reveal bilateral basilar fine crackles and digital clubbing may be present on examination of the fingers.[5,8] Non-specific symptoms include fatigue and weight loss. Pulmonary function tests (PFTs) will demonstrate restrictive ventilatory changes with reduced forced vital capacity (FVC), total lung capacity (TLC), and diffusing

capacity (DL_{CO}).[8] Complications of this disease process primarily involve the cardiopulmonary system consisting of respiratory failure, pulmonary hypertension, pulmonary embolism, heart attack, stroke, and lung cancer.

Clinical Phenotypes.

The heterogeneous clinical course of IPF can be used to classify three clinical phenotypes: stable or slow progressors, accelerated variants, and acute exacerbators.[8] A large majority of IPF patients are slow progressors who are often diagnosed after the disease has been established for years. Accelerated variants primarily consists of male cigarette smokers who experience a rapidly progressive clinical course. About 10% of IPF are acute exacerbators who experience rapid deterioration in their respiratory function in the absence of any identifiable cause.[8] The prognosis for acute exacerbators is very poor with > 60% mortality during hospital admission and > 90% mortality within 6 months after discharge.[9] Currently, disease progression is best monitored by PFTs, specifically focusing on FVC and DL_{CO}.[10] Additionally, DL_{CO} is a more reliable predictor of survival than FVC - a baseline DL_{CO} of < 40% predicted is associated with high mortality.[10]

Criteria for Diagnosis.

In 2011, a statement regarding the evidence-based diagnosis and management of IPF was released as a collaborative effort by the American Thoracic Society (ATS), the European

Respiratory Society (ERS), the Japan Respiratory Society (JRS), and the Latin American Thoracic Association (ALAT).[10] Briefly, it concluded that the diagnosis of IPF requires the exclusion of known causes of ILDs and the presence of usual interstitial pneumonia (UIP) on HRCT and/or surgical lung biopsy. On HRCT, the UIP pattern is observed as basilar reticular opacities (often with traction bronchiectasis) and sub-pleural honey-combing.[10] From a lung biopsy, the histological pattern of UIP is seen as areas with variable fibrosis intermixed with normal areas of parenchymal architecture with minimal inflammation. Additionally, the presence of myofibroblastic foci (areas of myofibroblast proliferation) are consistent findings that is currently the only histopathological marker that predicts mortality. The findings of UIP on HRCT are associated with a positive predictive value of 90-100% in biopsies positive with the UIP pattern.[10]

Current Treatment Options.

Currently, there is no evidence-based support for the use of any particular pharmacological agent in the treatment of IPF in the United States (**Table 2.2**). The collaborative efforts of ATS/ERS/JRS/ALAT has made strong recommendations against most treatments due to the lack of evidence supporting their benefits. These medications include but are not limited to corticosteroids with or without immuno-modulators (azathioprine or cyclophosphamide), colchicine, cyclosporine, IFN-γ, bosentan, and etanercept.[10] In certain sub-populations of IPF patients, certain therapies may be appropriate in their management such as

the usage of N-acetylcysteine with or without azathioprine and prednisone, and anti-coagulants. However, since the publication of these guidelines, results from the PANTHER-IPF clinical trial (double-blinded, randomized, placebo-controlled) demonstrated significant excess of deaths (11% versus 1%), hospitalizations (29% versus 8%), and adverse effects (31% versus 9%) with triple therapy (prednisone, azathioprine, and N-acetylcysteine) compared to placebo resulting in its early termination.[11] Similarly, the ACE-IPF trial demonstrated that warfarin was also associated with a significant increase risk of mortality in IPF patients (19%) compared to placebo (4%).[12] The collaboration also strongly recommends that IPF patients experiencing hypoxemia at rest should receive long-term oxygen therapy. Additionally, lung transplantation is strongly recommended in appropriate patients. With the lack of treatment, symptomatic control is appropriate as part of managing IPF patients.[10] In the United Kingdom, the National Institute for Health and Care Excellence recommends the use of pirfenidone for IPF patients with a predicted FVC between 50-80%.[13] Pirfenidone is an oral drug with anti-fibrotic, anti-inflammatory, and anti-oxidant properties. However due to conflicting results from clinical trials, the use of pirfenidone has not been approved for treating IPF in the United States.

Table 2.2. Summary of treatment recommendations made by ATS/ERS/JRS/ALAT in 2011.

STRONG RECOMMENDATION AGAINST USAGE	
MEDICATION	**MECHANISM OF ACTION**
Corticosteroids ± azathioprine or cyclophosphamide	Anti-inflammatory and immuno-suppression (purine analog and alkylating agent)
Colchicine	↓ lactic acid and uric acid and anti-inflammatory
Cyclosporine A	Immuno-suppression (calcineurin/NFAT inhibitor)
IFN-γ	Immunomodulator with anti-fibrotic properties
Bosentan	Endothelin receptor antagonist
Etanercept	Recombinant TNF-α receptor
Ambrisentan	Endothelin A receptor antagonist
WEAK RECOMMENDATION AGAINST USAGE	
Acetylcysteine monotherapy	Anti-oxidant
Prednisone + azathioprine + N-acetylcysteine*	Anti-inflammatory, immuno-suppression, anti-oxidant
Warfarin*	Anti-coagulant
Pirfenidone	Anti-fibrotic, anti-inflammatory, anti-oxidant

* Results from clinical trials published after 2011 indicated that these treatment options were either ineffective and/or associated with higher risk of mortality and adverse effects.[11-12, 14]

Clinical Trials and Future Treatment Options.

Due to the lack of effective treatment options for IPF in the United States, various clinical trials based on novel findings in the pathogenesis of IPF are currently being pursued (**Table 2.3**). Currently, there is a debate on what parameter is the most clinically meaningful end-point to use in these clinical trials. A clinically meaningful end-point directly reflects the patients' symptoms, functions, and survival.[15] Raghu et al. proposed that the best end-points to

use would be all-cause mortality and all-cause hospitalization.[15] However, in order for these end-points to reach statistical significance, a large sample population with longer follow-up time is required, both of which are difficult to achieve due to the high costs.[16] Alternative and more feasible markers or "surrogate end-points," for mortality include serial changes in FVC, DL_{CO}, and the six-minute walk test (6MWT) or progression-free survival.[15-17] In the clinical setting, some features are associated with higher mortality in IPF patients and can be used to help monitor the progression of the disease (**Table 2.4**).

Table 2.3A. Brief summary of some of the diverse completed clinical trials for IPF.[20]

COMPLETED CLINICAL TRIALS		
REGISTRATION #	**TREATMENT**	**RESULTS/NOTES**
NCT00600028	Thalidomide	Improved cough and respiratory quality of life[18]
NCT00903331	Macitentan	MUSIC trial; RCT phase II; oral endothelin antagonist; primary end-point in change in FVC in 1 year not met[19]
NCT00074698	FG-3019	CTGF monoclonal antibody
NCT00391443	Bosentan	No change from placebo[20]
NCT00131274	Gleevec/Imatinib	No effect on survival or lung function[21]
NCT00262405	Zileuton	Vs. azathioprine/prednisone
NCT00463983	Octreotide	Somatostatin analogue; stable lung function but non-randomized, non-controlled study[22]
NCT00125385	Fresolimumab/ GC-1008	Pan TGF-β antibody - results not published
NCT00786201	CNTO-888	Anti-CCL-2 monoclonal antibody - results not published
NCT01362231	AB-0024/GS-6624	Anti-LOXL-2 monoclonal antibody - results not published

MUSIC = Macitentan Use in an Idiopathic Pulmonary Fibrosis Clinical Trial; RCT = randomized control trial; FVC = forced vital capacity; CTGF = connective tissue growth factor; TGF-β = transforming growth factor-beta; CCL-2 = chemokine (C-C motif) ligand-2; LOXL-2 = lysyloxidase-like-2 protein; FG = FibroGen; GC = Genzyme; CNTO = Centocor; AB/GS = Gilead.

Table 2.3B. Brief summary of some IPF trials that are recruiting patients.[20]

RECRUITING FOR CLINICAL TRIALS		
REGISTRATION #	**TREATMENT**	**MECHANISM OF ACTION**
NCT01777737	Cotrimoxazole	Antibiotic (trimethoprim + sulfamethaoxazole)*
NCT01872689	Lebrikizumab	Anti-IL-13 monoclonal antibody
NCT01766817	BMS-986020	Lysophospatidic acid receptor antagonist
NCT01371305	STX-100	Anti-αvβ6 integrin monoclonal antibody

* No effect on lung function but improved quality of life in a UK study[23]; IL = interleukin; BMS = Bristol-Myers-Squibb; STX = Stromedix.

Table 2.3C. Brief summary of some terminated IPF clinical trials.[20]

TERMINATED CLINICAL TRIALS		
REGISTRATION #	**TREATMENT**	**RESULTS**
NCT00703339	Treprostinil sodium	Used for PHT; unknown due to lack of enrollment for the study[24]
NCT00879229	Ambrisentan	Used for PHT; ARTEMIS-IPF, RCT; lack of benefit and increased hospitalization[25]
NCT01203943	CC-930	JNK inhibitor; benefit/risk profile does not support continuation[24]
NCT01266135	QAX-576	Anti-IL-12 mAB
NCT00517933	Sildenafil	STEP-IPF, DRCT; primary end-point in 6MWT not met but DL_{CO} was improved[26]
NCT01462006	Sirolimus/Rapamycin	mTOR inhibitor

PHT = pulmonary hypertension; RCT = randomized control trial; JNK = c-Jun-N-terminal kinase; IL = interleukin; DRCT = double RCT; 16MWT = 6 minute walk test; DL_{CO} = diffusing capacity of the lung for carbon monoxide; mTOR = mechanistic target of rapamycin.

Table 2.4. Clinical features associated with higher mortality in IPF patients (adapted from ATS).[10]

BASELINE FACTORS	LONGITUDINAL FACTORS
Level of dyspnea	Increase in level of dyspnea
DL_{CO} < 40% predicted	Decrease in DL_{CO} ≥ 15% absolute value
Desaturation ≤ 88% during 6MWT	Decrease in FVC ≥ 10% absolute value
% honey-combing on HRCT	Worsening of fibrosis on HRCT
Pulmonary Hypertension	

Familial Pulmonary Fibrosis.

Familial Pulmonary Fibrosis (FPF), also known as Familial Interstitial Pneumonia, accounts for < 5% of IPF cases and is clinically indistinguishable from sporadic cases except for the occurrence of an IIP in ≥ 2 first-degree biological relatives and the possibility of earlier age of onset.[27] Currently, it is believed that this disease is inherited as an autosomal-dominant trait with variable penetrance.[27] Mutations in the following genes are associated with the risk of developing FPF: telomerase-related genes, *TERT* and *TERC*, surfactant proteins, *SP-C* and *SP-A,* and a mucin gene, *MUC5B*. Mutations in FPF suggests underlying genetic components in IPF (**Table 2.5**). The identification of telomerase-related genes as possible candidates for FPF was influenced by the association of these mutations in Hermansky-Pudlak Syndrome and dyskeratosis congenita (clinical syndromes with pulmonary fibrosis).[28] Mutations in *TERT* and *TERC* are associated with telomere shortening resulting in cell death due to chromosomal instability.[29]

Interestingly, the other affected genes are expressed in lung epithelial cells, with *SP-C* and *SP-A* being unique to type II alveolar epithelial cells (AECs). Mutations in *SP-C* mainly reside in the BRICHOS domain which normally functions to prevent protein aggregation during insertion into the membrane.[31] Whole-genome linkage analysis in a cohort of 59 kindreds with FPF identified two missense mutations in the highly conserved carbohydrate recognition domain in *SP-A2*, G231V and F198V.[32] SP-A belong to the family of collectins, innate-immune defense proteins and these mutations are predicted to result in their instability and accumulation in the endoplasmic reticulum (ER).[32] Both *SP-C* and *SP-A* mutations are hypothesized to result in ER-stress induced apoptosis of AECs due to the activation of the unfolded protein response (UPR) from the accumulation of misfolded proteins.[33] The common promoter polymorphism in *MUC5B* (rs35705950) leads to AEC injury by three proposed mechanisms: 1) the *MUC5B* variant leads to excess mucin production that impairs mucosal host defense and effective ciliary movement for clearance, 2) the over-production of mucin impairs alveolar repair, and 3) the ectopic production of mucin leads to heterogeneity in fibrosis.[34] *MUC5B* encodes a major gel-forming mucin found in the mucous secretions of saliva, the lung, and the cervix. Surprising, this common mutation is also associated with increase survival in IPF.[35] In addition to the aforementioned genes, other genes have been implicated in IPF as well but require further validation studies (**Table 2.6**).

Table 2.5. Genes implicated in the pathogenesis of FPF and IPF with estimated percentage of genetic contributions to the disease.

FPF	GENE	IPF
34%	*MUC5B*[33]	38%
17%	*TERT* and *TERC*	3%
< 1%	*SP-C*	< 1%
< 1%	*SP-A*	< 1%
47%	UNKNOWN	57%

FPF = familial pulmonary fibrosis; IPF = idiopathic pulmonary fibrosis; MUC5B = mucin 5B; TERT = telomerase reverse transcriptase; TERC; telomerase RNA component; SP = surfactant protein.

Table 2.6. Other genes implicated in the progression and/or survival in IPF.

GENERAL FUNCTION	GENE
CYTOKINES	IL-1α, IL-4, IL-6, IL-8, IL-10, IL-12
	TNF-α
	Lymphotoxin α
	TGF-β_1
ENZYMES	α_1-anti-trypsin
	MMP-1
	SPPL-2C
COAGULATION PATHWAY	PAI-1, PAI-2
SURFACTANT PROTEINS	SP-B, SP-D
IMMUNOMODULATORY	Complement receptor 1
	NOD2
	HLA-A, HLA-B
	MHC class I chain-related genes
	TOLLIP
	TLR-3
	CXCL-5
	CXCL-10
ANGIOTENSIN SYSTEM	AGT
	ACE
EICOSANOID PATHWAY	COX-2
microRNA	miR-199a-5p
FOLATE PATHWAY	Transcobalamin II
MITOGENS	VEGF
DESMOSOMES	DSP

Prevailing Hypothesis Underlying IPF: Abnormal Wound Healing

Key players in Fibrosis: Alveolar Epithelial Cells and Myofibroblasts.

95% of the alveolar surface area is composed of squamous type I AECs while the remaining 5% is composed of cuboidal type II AECs.[36] However, this discrepancy in surface area composition is not reflected in absolute cell numbers - type II AECs represent 60% of epithelial cells lining the alveoli, however due to their cuboidal morphology, they cover less surface area.[36] Type I AECs primarily function in gas exchange while type II AECs have the capacity for regeneration and are the site of production of pulmonary surfactant.[37] Pulmonary surfactant lowers surface tension within the alveoli to help assist with gas exchange. These pneumocytes are often found at the corners and intersections of alveolar walls in pairs or triplets. The airway epithelium functions as a physical barrier against foreign particles and microbes and also in gas exchange.

Myofibroblasts are contractile cells that are well known sources of collagen and an active participant in wound repair. They can be derived from pericytes, fibrocytes, epithelial cells, or resident fibroblasts - however, these sources are still under debate. The proximity of resident fibroblasts and myofibroblasts in addition to the activation of fibroblasts by chemokines and cytokines from the nearby environment supports the migration and differentiation of these local fibroblasts into myofibroblasts.[38] In addition to being derived from resident fibroblasts, myofibroblasts can also be generated from circulating CXCR-4 positive fibrocytes which are attracted to the lung by high expression of CXCL-12 from the epithelial

cells.[39] Compared with healthy controls, IPF patients were found to have higher numbers of circulating fibrocytes in the blood.[39] Lastly, epithelial-to-mesenchymal transition (EMT) can also be a source of myofibroblasts. In this process, the epithelial cell phenotype (E-cadherin) is lost as the cell gains a mesenchymal phenotype (α-smooth muscle actin and fibronectin).[40]

Abnormal Wound Healing.

The prevailing hypothesis underlying the pathogenesis of IPF is that it is a result of abnormal wound healing.[41] Abnormal wound healing consists of persistent injury to AECs, aberrant fibroblast proliferation and the accumulation of extracellular matrix proteins.[41] Injury to the alveolar epithelium is the initiating factor. Both environmental and genetic insults have been implicated in this process. It is hypothesized that genetic mutations mediate AECs injury through ER- stress and the induction of the UPR (see **Chapter 5**). To replace these damaged cells, type II AECs becomes hyperplastic. In the normal repair process, these hyperplastic AECs will undergo regulated apoptosis and the remaining AECs will differentiate into type I AECs. However, in pathologic conditions, these hyperplastic cells remain leading to the activation of TGF-β_1.

Role of TGF-β_1 in Fibrosis.

TGF-β_1 is one of three mammalian isoforms in the TGF-β superfamily of cytokines. In pulmonary fibrosis, this is the best characterized isoform and is the main cytokine implicated in

the fibrotic response.[42] TGF-β_1 induces the migration and apoptosis of epithelial cells, EMT, collagen synthesis, and the proliferation and differentiation of fibroblasts.[42] In tissue biopsies from IPF patients, both TGF-β_1 protein and mRNA are up-regulated compared to healthy controls.[43-44] A variety of cells in the lung are sources of TGF-β, including alveolar macrophages, AECs, endothelial cells, fibroblasts, and myofibroblasts.[45]

TGF-β_1 is secreted by most cells as an inactive form bound by a latency-associated peptide (LAP) and latent TGF-β binding proteins (LTBPs). In order to be activated, TGF-β_1 must alter its interaction with the LAP. This alteration can be mediated through various mechanisms including physical disturbances (such as temperature extremes, low pH, and oxidation), proteases (such as plasmin, tryptase, thrombin, and elastase), or interactions with integrins. The latter of which is important in pulmonary fibrosis, particularly with the $\alpha_v\beta_6$ integrin.[42] The integrin-mediated activation of TGF-β_1 involves a conformational change induced by the binding of $\alpha_v\beta_6$ to the Arginine-Glycine-Aspartic Acid (RGD) sequence in the LAP in addition to a tensile force generated by the contraction of the cell (**Figure 2.2**).[46]

TGF-β signaling can be mediated through the classical canonical pathway or the non-canonical pathway. The signaling from both pathways are initiated upon binding of TGF-β ligands to the type II TGF-β receptor (TβRII) that will dimerize with a type I TGF-β receptor (TβRI).[47] In the classical pathway, TβRI phosphorylates SMAD-2/3 allowing for its interaction with SMAD-4.[47] This SMAD complex will enter the nucleus to regulate the transcription of genes. In the non-canonical pathway, recruitment of TGF-β activated kinases (TAK-1) and TAK-1

binding protein (TAB) activates mitogen-activated protein kinase (MAPK) cascades, such as the c-Jun N-terminal kinase (JNK) and p38 kinase pathways.[48] Both pathways are implicated in IPF. For instance, the Fas-mediated apoptosis of AECs induced by TGF-β involves JNK/MAPK signaling whereas both SMAD-dependent and SMAD-independent pathways are involved in TGF-β-induced EMT.[45]

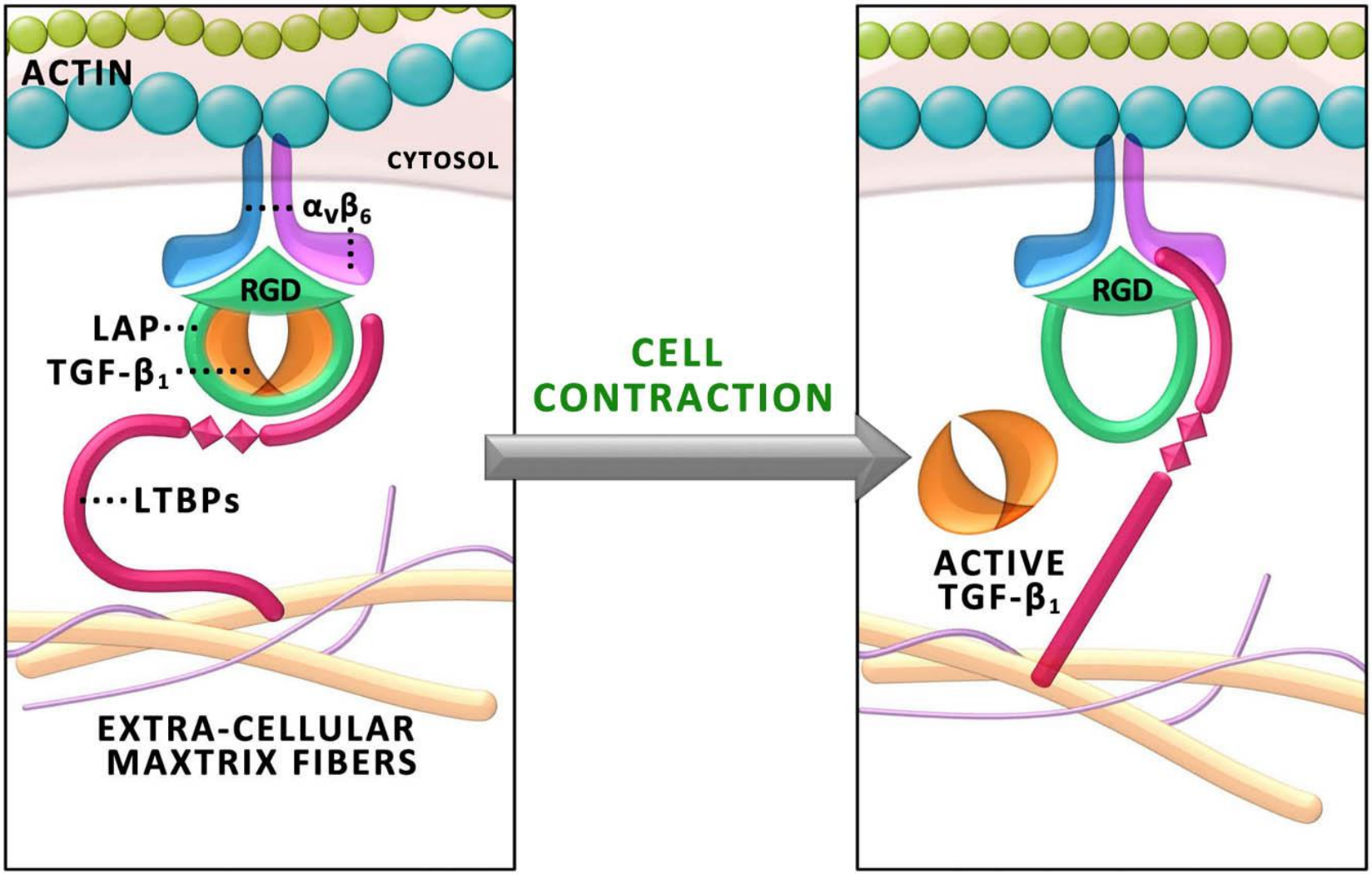

Figure 2.2. Integrin-mediated activation of TGF-β_1. The $\alpha_v\beta_6$ intergin plays an important role in pulmonary fibrosis. RGD = arginine-glycine-aspartic acid motif; LAP = latency-associated peptide; LTBPs = latent TGF-β_1 binding proteins.

Conclusions

IPF is the most common form of ILD with an estimated prevalence of about 50,000 in the United States.[5] Currently, there are no medications that have been shown to slow the progression of this disease. Consequently, there are no evidence-based medical support for the use of any particular drug in treating IPF in the United States. Treatment of IPF is centered around supportive therapy, which consists of the use of oxygen, pulmonary rehabilitation, lung transplantation, and agents to combat cough and gastroesophageal reflux disease (GERD) - the latter of which is a common co-morbidity present in IPF.[4] However, the option for lung transplantation is only available to a minority of patients and the five year post-operative survival rate is only 44%.[5]

The lack of effective therapies stems from our incomplete understanding the pathogenesis of this disease. Additionally, due to the rarity of this disease, IPF is designated as an orphan disease resulting in minimal financial incentive for pharmaceutical companies to develop new therapies in treating this disease. Due to the heterogeneous nature of this disease, experts at the International Colloquium for Lung and Airway Fibrosis recognized the need to identify phenotypes in order to sub-classify IPF. To overcome these hurdles, I propose that we should shift our focus away from the idea that IPF is an inflammatory disease to the idea that IPF is a result of abnormal wound healing.[49] In IPF, inflammation is not a major histopathological finding and is not required for fibrosis - epithelial injury with the absence of inflammation is sufficient to induce fibrosis.[50] Similarly, markers of inflammation are not

correlated with the fibrotic response.[50] Lastly, the use of anti-inflammatories (such as corticosteroids) as traditional "standard therapies" failed to show benefits in IPF patients. Similarly, the use of prednisone, azathioprine, and N-acetylcysteine that were formerly used as the "standard triple therapy" was not only deemed to be ineffective but also detrimental. In the PANTHER-IPF clinical trial, IPF patients on this triple therapy regimen experienced greater adverse effects, hospitalizations, and death compared to the placebo group.[11]

If we can re-purpose FDA-approved drugs to treat this disease, additional resources would not have to be invested in developing a new one. Since the therapeutic profile of FDA-approved drugs are well-known, less optimization would be required in implementing them in a clinical trial compared to a new one. Finally, biomarkers can be used as genetic phenotypes to help sub-classify the population of IPF. In the following chapters, these solutions will be addressed by focusing on the roles of the ANG system in the abnormal wound healing process and the use of *AGT* variants as predictors of severity in IPF. Both providing support for the use of ARBs as therapeutic agents in treating IPF.

REFERENCES

REFERENCES

1. Peroš-Golubičić, T., and Sharma, O.P. Definition, Classification, and Clinical Features in *Clinical Atlas of Interstitial Lung Disease.* Edited by Peroš-Golubičić, T., Sharma, O.P., and King, T.E. 2.7-2.17 (Springer-Verlag, London, France, 2006).

2. Baughman, R.P., Montanaro, F., and Bottino, G. Interstitial Lung Disease: Introduction in *Pulmonary Arterial Hypertension and Interstitial Lung Diseases, A Clinical Guide.* Edited by Baughman, R.P., Carbone, R.G., and Bottino, G. 1.3-1.12 (Humana Press, New York, USA 2009).

3. Alhamad, E.H., and Cosgrove, G.P. Interstitial Lung Disease: The Initial Approach. *Med. Clin. N. Am.* 95:1071-1093 (2011).

4. Travis, W.D., Costabel, U., Hansell, D.M., Talmadge, K.E., Lynch, D.A., Nicholson, A.G., Ryerson, C.J., Ryu, J.H., Selman, M., Wells, A.U., Behr, J., Bouros, D., Brown, K.K., Colby, T.V., Collard, H.R., Cordeiro, C.R., Cottin, V., Crestani, B., Drent, M., Dudden, R., Egan, J., Flaherty, K., Hogaboam, C., Inoue, Y., Johkoh, T., Kim, D.S., Kitaichi, M., Loyd, J., Martinez, F.J., Myers, J., Protzko, S., Raghu, G., Richeldi, L., Sverzellati, N., Swigris, J., and Valeyre, D. An official American Thoracic Society/European Respiratory Society Statement: update of the international multidisciplinary classification of the idiopathic interstitial pneumonias. *Am. J. Respir. Crit. Care Med.* 188:733-748 (2013).

5. King, T.E. Jr., Pardo, A., and Selman, M. Idiopathic pulmonary fibrosis. *Lancet.* doi:10.1016/20140-6736(11)60052-4 (2011).

6. Taskar, V.S., and Coultas, D.B. Is idiopathic pulmonary fibrosis an environmental disease? *Proc. Am. Thorac. Soc.* 3:293-298 (2006).

7. Baumgartner, K.B., Samet, J.M., Stidley, C.A., Colby, T.V., and Waldron, J.A. Cigarette smoking: a risk factor for idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 155:284-289 (1996).

8. Peroš-Golubičić, T., and Sharma, O.P. Idiopathic Pulmonary Fibrosis in *Clinical Atlas of Interstitial Lung Disease.* 6.41-6.45 (Springer-Verlag, London, 2006).

9. Song, J.W., Hong, S.B., Lim, C.M., Koh, Y., and Kim, D.S. Acute exacerbation of idiopathic pulmonary fibrosis: incidence, risk factors and outcome. *Eur. Respir. J.* 37:356-363 (2011).

10. Raghu, G., Collard, H.R., Egan, J.J., Fernando, J.M., Behr, J., Brown, K.K., Colby, T.V., Cordier, J.F., Flaherty, K.R., Lasky, J.A., Lynch, D.A., Ryu, J.H., Swigris, J.J., Wells, A.U., Ancochea, J., Bouros, D., Carvalho, C., Costabel, U., Ebina, M., Hansell, D.M., Johkoh, T., Kim, D.S., King, T.E., Kondoh, Y., Myers, J., Müller, N.L., Nicholson, A.G., Richeldi, L., Selman, M., Dudden, R.F., Griss, B.S., Protzko, S.L., and Schünemann, H.J. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am. J. Respir. Crit. Care Med.* 183:788-824 (2011).

11. NIH News. Commonly used three-drug regimen for idiopathic pulmonary fibrosis found harmful. Bethesda, National Institutes of Health (2011). www.nih.gov/news/health/oct2011/nhlbi-21.htm

12. Noth, I., Anstrom, K.J., Calvert, S.B., de Andrade, J., Flaherty, K.R., Glazer, C., Kaner, R.J., and Olman, M.A. A placebo-controlled randomized trial of warfarin in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 186:88-95 (2012).

13. NICE guidance on pirfenidone for treating idiopathic pulmonary fibrosis. *Lancet.* 1:191-192 (2013).

14. Antoniou, K.M., Margaritopoulous, G.A., and Siafakas, N.M. Pharmacological treatment of idiopathic pulmonary fibrosis: from the past to the future. *Eur. Respir. Rev.* 22:281-291 (2013).

15. Raghu, G., Collard, H.R., Anstrom, K.J., Flaherty, K.R., Fleming, T.R., King, T.E., Martinez, F.J., and Brown, K.K. Idiopathic pulmonary fibrosis: clinically meaningful primary endpoints in phase 3 clinical trials. *Am. J. Respir. Crit. Care Med.* 185:1044-1048 (2012).

16. Wells, A.U., Behr, J., Costabel, U., Cottin, V., Poletti, V., and Richeldi, L. Hot of the breath: mortality as a primary end-point in IPF treatment trials: the best is the enemy of the good. *Thorax.* 67:938-940 (2012).

17. Vancheri, C., du Bois, R.M. A progression-free end-point for idiopathic pulmonary fibrosis trials: lessons from cancer. *Eur. Respir. J.* 41:262-269 (2013).

18. Horton, M.R., Santopietro, V., Mathew, L., Horton, K.M., Polito, A.J., Liu, M.C. Danoff, S.K., and Lechtzin, N. Thalidomide for the treatment of cough in idiopathic pulmonary fibrosis: a randomized trial. *Ann. Intern. Med.* 157:398-406 (2012).

19. Raghu, G., Million-Rousseau, R., Morganti, A., Perchenet, L., and Behr, J. Macitentan for the treatment of idiopathic pulmonary fibrosis: the randomised controlled MUSIC trial. *Eur. Respir. J*. 42:1622-1632 (2013).

20. King, T.E., Brown, K.K., Raghu, G., du Bois, R.M., Lynch, D.A., Martinez, F., Valeyre, D., Leconte, I., Morganti, A., Roux, S., and Behr, J. BUILD-3: a randomized, controlled trial of bosentan in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 184:92-99 (2011).

21. Daniels, C.E., Lasky, J.A., Limper, A.H., Mineras, K., Gabor, E., and Schroeder, D.R. Imatinib treatment for idiopathic pulmonary fibrosis: randomized placebo-controlled trial results. *Am. J. Respir. Crit. Care Med.* 181:604-610 (2010).

22. Crestani, B., Chapron, J., Wallaert, B., Bergot, E., Delaval, P., Israel-Biet, D., Lacronique, J., Reynaud-Gaubert, M.M., Tazi, A., Lebtahi, R., Debray, M.P., Brauner, M. Dehoux, M., Dornic, Q., Aubier, M., Mentré, F., and Duval, X. Octreotide treatment of idiopathic pulmonary fibrosis: a proof-of-concept study. *Eur. Respir. J.* 39:772-775 (2012).

23. Shulgina, L., Cahn, A.P., Chilvers, E.R., Parfrey, H., Clark, A.B., Wilson, E.C., Twentyman, O.P., Davison, A.G., Curtin, J.J., Crawford, M.B., and Wilson, A.M. Treating idiopathic pulmonary fibrosis with the addition of co-trimoxazole: a randomized controlled trial. *Thorax.* 68:155-162 (2013).

24. ClinicalTrials.gov: Idiopathic Pulmonary Fibrosis. U.S. National Institutes of Health. http://www.clinicaltrials.gov/ct2/results?term=idiopathic+pulmonary+fibrosis+&Search=Search. Last visited May 2014.

25. Raghu, G., Behr, J., Brown, K.K., Egan, J., Kawut, S.M., Flaherty, K.R., Martinez, F.J., Nathan, S.D., Wells, A., Pedersen, P, Shao, L, Chien, J., and O'Riordan, T. Artemis-IPF: a placebo-controlled trial of ambrisentan in idiopathic pulmonary fibrosis. *B93 Presented at the American Thoracic Society Meeting in the Clinical Trials In Idiopathic Pulmonary Fibrosis and Sarcoidosis Section*. May 1, 2012; A3632.

26. Zisman, D.A., Schwarz, M., Anstrom, K.J., Collard, H.R., Flaherty, K.R., and Hunninghake, G.W. A controlled trial of sildenafil in advanced idiopathic pulmonary fibrosis. *N. Engl. J. Med.* 363:620-628 (2010).

27. Talbert, J.L., and Schwartz, D.A. Pulmonary Fibrosis, Familial from GeneReviews [Internet]. 2010.

28. Mason, P.J., and Bessler, M. The genetics of dyskeratosis congenita. *Cancer Genet.* 2204:635-645 (2011).

29. Artandi, S.E., and DePinho, R.A. A critical role for telomeres in suppressing and facilitating carcinogenesis. *Curr. Opin. Genet. Dev.* 10:39-46 (2000).

30. Wolters, P.J., Collard, H.R., and Jones, K.D. Pathogenesis of idiopathic pulmonary fibrosis. *Annu. Rev. Pathol. Mech. Dis.* 9:157-179 (2014).

31. Willander, H., Askarieh, G., Landreh, M., Westermark, P., Nordling, K., Keränen, H., Hermansson, E., Hamvas, A., Nogee, L.M., Bergman, T., Saenz, A., Casals, C., Âqvistg, J., Jörnvall, H., Berglund, H., Presto, J., Knight, S.D., and Johansson, J. High-resoultion structure of a BRICHOS domain and its implications for anti-amyloid chaperone activity on lung surfactant protein C. *Proc. Natl. Acad. Sci. USA.* 109:2325-2329 (2012).

32. Wang, Y., Kuan, P.J., Xing, C., Cronhkite, J.T., Torres, F., Rosenblatt, R.L., DiMaio, J.M., Kinch, L.N., Grishin, N.V., and Garcia, C.K. Genetic defects in surfactant protein A2 are associated with pulmonary fibrosis and lung cancer. *Am. J. Hum. Genet.* 84:52-59 (2009).

33. Noble, P.W., Barkauskas, C.E., and Jiang, D. Pulmonary fibrosis: patterns and perpetrators. *J. Clin. Invest.* 122:2756-2762 (2012).

34. Seibold, M.A., Wise, A.L., Speer, M.C., Steele, M.P., Brown, K.K., Loyd, J.E., Fingerlin, T.E., Zhang, W., Gudmundsson, G. Groshong, S.D., Evans, C.M., Garantziotis, S., Adler, K.B., Dickey, B.F., du Bois, R.M., Yang, I.V., Herron, A., Kervitsky, D., Talbert, J.L., Markin, C., Park, J., Crews, A.L., Slifer, S.H., Auerbach, S., Roy, M.G., Lin, J., Hennessy, C.E., Schwarz, M.I., and Schwartz, D.A. A common *MUC5B* promoter polymorphism and pulmonary fibrosis. *N. Engl. J. Med.* 364:1503-1512 (2011).

35. Peljto, A.L., Zhang, Y., Fingerlin, T.E., Ma, S.F., Garcia, J.G., Richards, T.J., Association between the MUC5B promoter polymorphism and survival in patients with idiopathic pulmonary fibrosis. *JAMA.* 309:2232-2239 (2013).

36. Barrett, K.E., Boitano, S., Barman, S.M., and Brooks, H.L. Chapter 34. Introduction to Pulmonary Structure and Mechanics in *Ganong's Review of Medical Physiology*. (McGraw-Hill Companies, China, 2012).

37. Mescher, A.L. Chapter 17: The Respiratory System in *Junqueira's Basic Histology Text and Atlas 13e* (McGraw-Hil Companies, China, 2013).

38. Selman, M., and Pardo, A. Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proc. Am. Thorac. Soc.* 3:413-417 (2006).

39. Andersson-Sjoland, A., de Alba, C.G., Nihlberg, K., Becerril, C., Ramírez, R., Pardo, A., Westergren-Thorsson, G., and Selman, M. Fibrocytes are a potential source of lung fibroblasts in idiopathic pulmonary fibrosis. *Int. J. Biochem. Cell. Biol.* 40:2129-2140 (2008).

40. Willis, B.C., duBois, R.M., and Borok, Z. Epithelial origin of myofibroblasts during fibrosis in the lung. *Proc. Am. Thorac. Soc.* 3:3777-3782 (2006).

41. Selman, M., King, T.E., and Pardo, A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann. Intern. Med.* 134:136-151 (2001).

42. Coward, W.R., Saini,, G., and Jenkins, G. The pathogensis of idiopathic pulmonary fibrosis. *Ther. Adv. Respir. Dis.* 4:367-388 (2010).

43. Khalil, N., O'Connor, R.N., Flanders, K.C., and Unruh, H. TGF-β1, but not TGF-β2 or TGF-β3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. *Am. J. Respir. Cell. Mol. Biol.* 14:131-138 (1996).

44. Coker, R.K., Laurent, G.J., Jeffery, P.K., du Bois, R.M., Black, C.M., and McAnulty, R.J. Localisation of transforming growth factor beta1 and beta3 mRNA transcripts in normal and fibrotic human lung. *Thorax.* 56:549-556 (2001).

45. Fernandez, I., and Eickelberg, O. The impact of TGF-β on lung fibrosis. *Proc. Am. Thorac. Soc.* 9: 111-116 (2012).

46. Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., and Springer, T.A. Latent TGF-β structure and activation. *Nature.* 474:343-351 (2011).

47. Derynck, R., and Zhang, Y.E. SMAD-dependent and SMAD-independent pathways in TGF-beta family signaling. *Nature.* 425:577-584 (2003).

48. Davis, J., and Molkentin, J. Myofibroblasts: Trust your heart and let fate decide. *J. Mol. Cell. Cardiol.* http://dx.doi.org/10.1016/j.yjmcc.2013.10.019 (2013).

49. Uhal, B.D., and Nguyen, H. The Witschi Hypothesis revisited after 35 years: Genetic proof from SP-C BRICHOS domain mutations. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 305:L906-911 (2013).

CHAPTER 3

TRANSCRIPTIONAL REGULATION OF *ANGIOTENSINOGEN* IN HUMAN LUNG FIBROBLASTS*

* Adapted from the co-authored paper entitled Angiotensinogen Gene Transcription in Pulmonary Fibrosis published in *Int. J. Pept*. 2012.

The ANG System in IPF

The ANGII-TGF-β1 Cross-Talk.

IPF is a result of abnormal wound healing. Injury to the alveolar epithelium is the initiating factor. Injured AECs transforms latent TGF-β_1 into its active form. Type II AECs are unable to replace the injured AECs and becomes hyperplastic. These hyperplastic AECs activate TGF-β_1. TGF-β_1 induces a profibrotic phenotype in fibroblasts by: 1) mediating the transformation of fibroblasts into myofibroblasts, 2) increasing pro-fibrotic genes [such as collagen and alpha-smooth muscle actin (α-SMA)], 3) suppressing the apoptosis of fibroblasts, and 4) inducing *AGT* transcription in fibroblasts to generate the pro-fibrotic peptide, ANGII.[1-2] ANGII induces the apoptosis of AECs, contributing to the repetitive injury of the alveolar epithelium. These injured AECs contribute to the aberrant fibroblast proliferation by activating TGF-β_1.

In human lung fibroblasts isolated from IPF patients, there is an over-expression of AGT mRNA and protein, TGF-β_1 mRNA and protein, and ANGII.[1] Previous work by our laboratory demonstrated an "autocrine TGF-β_1-ANGII loop" in fibroblasts.[1] In fibroblasts, ANGII enhances TGF-β_1 synthesis and in return, TGF-β_1 induces the transcription of *AGT*, thereby helping to generate the local pool of ANGII.[1] Additionally, TGF-β_1 stimulates the transformation of fibroblasts into myofibroblasts and their resistance to apoptosis. Both events contribute to the aberrant proliferation of fibroblasts and the accumulation of extracellular matrix proteins. Within many tissues, myofibroblasts are a known source of collagen and ANGII. Furthermore,

ANGII can mediate the apoptosis of AECs, further contributing to the development of lung fibrosis (**Figure 3.1**).

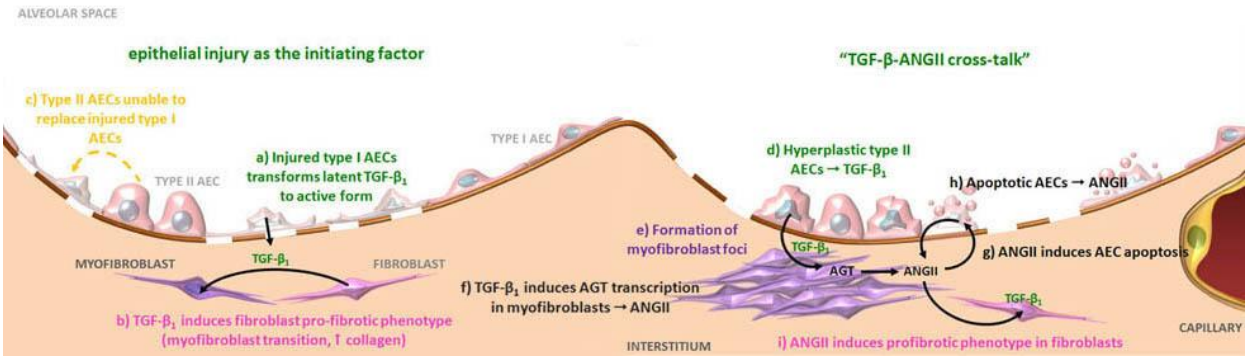

Figure 3.1. Role of the ANG system in abnormal wound healing.

Lung-Derived Angiotensin System.

Components of the renin-angiotensin system are expressed in various cells from the heart, brain, and lung.[3-6] In the lung, this system is independent from the classical renin-angiotensin-aldosterone endocrine system that is well known to regulate blood pressure. Instead of relying on renin and ACE for the enzymatic conversion of AGT to ANGI and ANGI to ANGII, respectively, other enzymes carry out this process. These enzymes include cathepsin D, tonin, cathepsin G, or chymase and are locally generated within the tissue with minimal contributions from circulating levels. In human pulmonary fibrosis, cathepsin D is up-regulated and is required for the apoptosis of AECs mediated by ANGII.[7-8]

In the lung, injured AECs and myofibroblasts are sources of AGT.[9-10] In primary cultures of myofibroblasts isolated from IPF biopsies, AGT mRNA and protein in addition to ANGII were detected.[10] IPF biopsies also demonstrated a 21-fold and 3.6 fold increase in AGT mRNA and

protein, respectively.[11] In response to apoptosis inducers, bleomycin, Fas ligand, or TNF-α, AGT was synthesized and processed into ANGII in primary cultures of AECs.[9, 12-13] Additionally, apoptosis in response to these inducers were dependent on *AGT* synthesis as apoptosis was blocked with antisense oligonucleotides against *AGT.*[9, 12-13] This suggests that *AGT* transcription is the rate-limiting step in the generation of ANGII. Additionally, the plasma concentration of AGT (1 μM) is similar to the Michaelis-Menten Constant of renin. Therefore, alterations in the concentration of AGT can influence the generation of ANGII.[14] For instance, in the bleomycin-induced pulmonary fibrosis model, the up-regulation of *AGT* in the lung led to an increase in ANGII.[15] Anti-sense oligonucleotides against *AGT* prevented bleomycin-induced collagen accumulation in cultured lung explants indicating the requirement of *AGT* in this process.[15]

Angiotensinogen Expression and Regulation.

AGT is a member of the serine protease inhibitor superfamily and functions as the starting substrate in the ANG system. This gene is encoded by 5 exons and 4 introns with the second exon containing the initiation Methionine, signal peptide, and the mature protein, ANGII (along with its pre-cleavage precursor, ANGI; **Figure 3.2A**).[16] Cleavage of AGT by renin or cathepsin D to generate ANGI occurs between Leucine^{10} - Valine^{11} - a site that is unique to primates (**Figure 3.2B**).[17] In addition to this, the substitution of Histidine^{13} to Tyrosine^{13} in human AGT contributes to the species specificity of this reaction.[18]

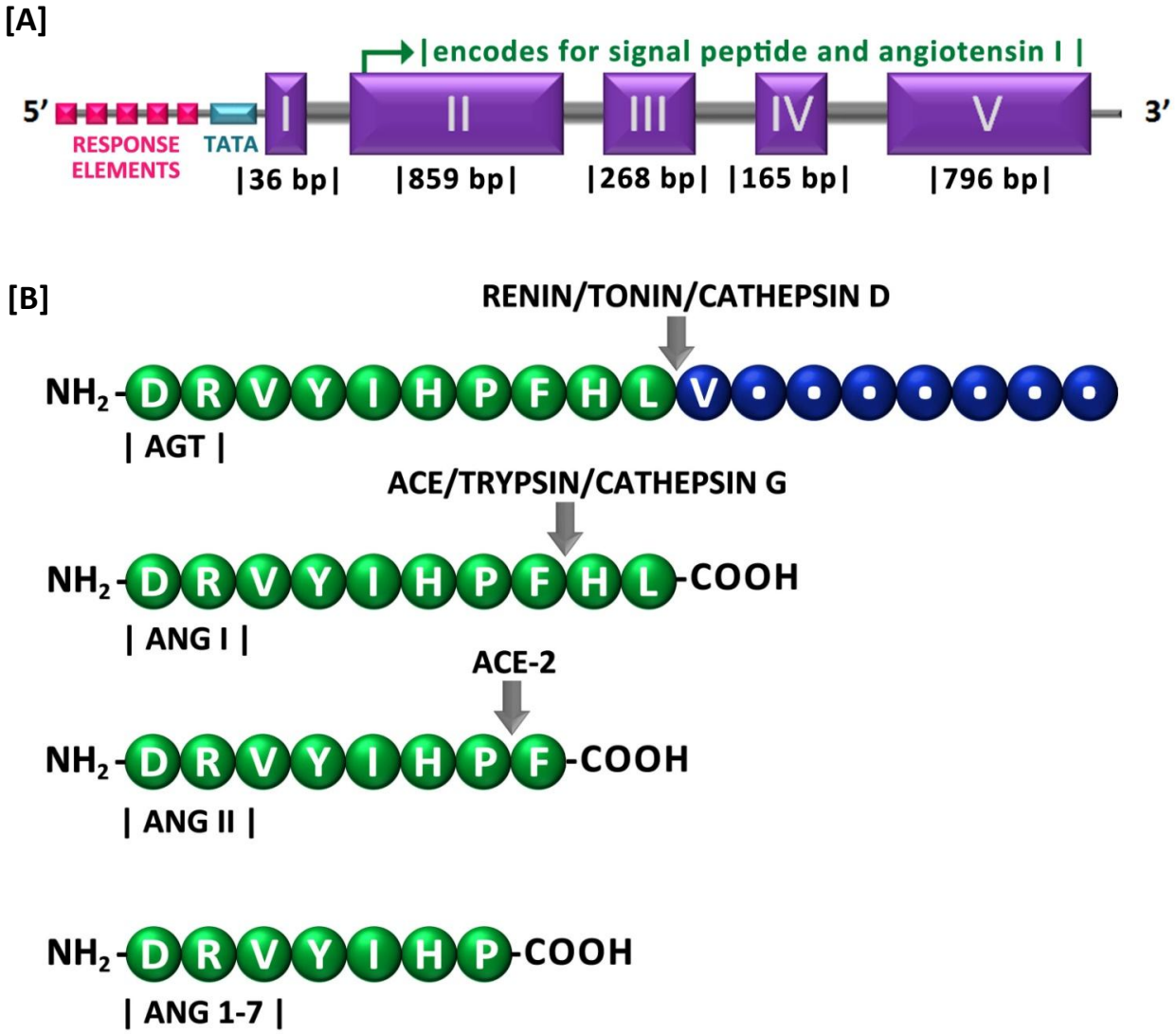

Figure 3.2. Organization of *AGT*. [A] The signal peptide and angiotensin I is encoded in exon II of *AGT.* **[B]** AGT serves as the precursor to ANG peptides that are generated from the cleavage of one or two amino acids from the carboxy-terminal.

The abundance of AGT is transcriptionally regulated and is influenced by hormonal and cell-type specific regulators.[19] For instance, direct repeat sequences [DRs (AGGTCA)] in the promoter (located at -431 to -380 and -281 to -252) contributes to *AGT* transcription in the liver (50%) and kidneys (95%) but is not required in the heart and brain.[20] Receptors to glucocorticosteroids, thyroid hormones, and estrogens can bind to these DRs to alter *AGT* synthesis - though it is not well known which combinations of these factors influence the tissue specificity of *AGT* expression.[21] In the liver, hepatocyte nuclear factor (HNF-4) is a candidate factor for these DRs in up-regulating *AGT* expression.[20, 22] *AGT* expression was inhibited in response to bile acids by a small heterodimer partner (SHP) binding to a similar site as HNF-4.[23] In hepatocytes, interleukin (IL-6) mediates *AGT* transcription through the binding of STAT-3, HNF-1α, and the glucocorticoid receptor to the promoter.[24-25] Interferon gamma (IFN-γ) mediated *AGT* transcription involves the binding of STAT-1 to a region between -271 and -279 in the promoter.[26]

In AECs, amiodarone (an anti-arrhythmic drug that causes pulmonary fibrosis as a side-effect) inducible *AGT* transcription is mediated by an AP-1 binding site located between the TATA box and transcription start site.[27] In pulmonary fibroblasts, TGF-β_1-inducible *AGT* transcription is mediated by JunD (an AP-1 transcription factor) and HIF-1α. The binding sites for AP-1 and HIF-1α overlap known SNPs in the core promoter (**Figure 3.3**).[28] SNPs located at the -20, -18, and -6 positions have been shown to alter the rate of *AGT* transcription in hepatocytes.[29-30]

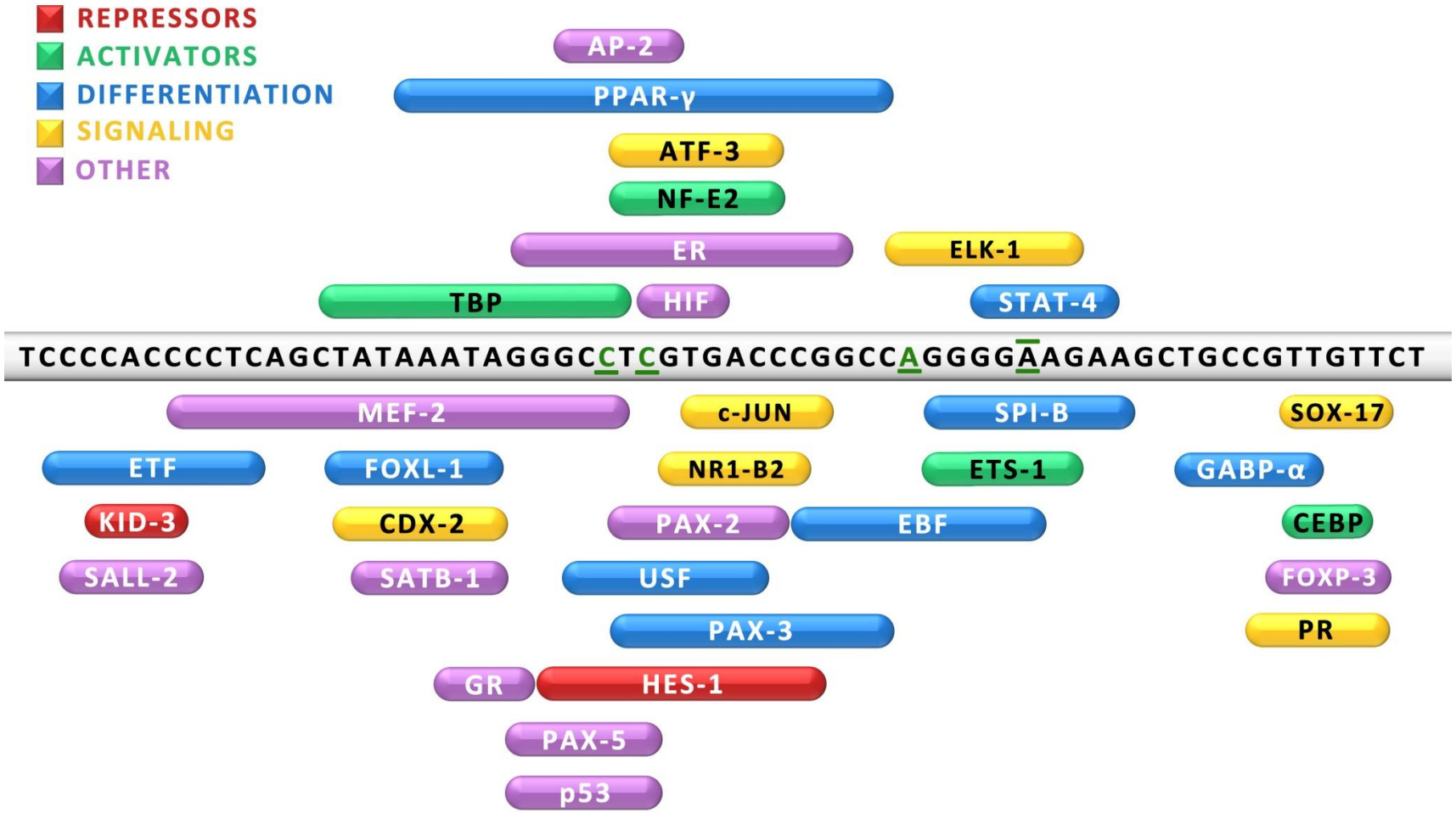

Figure 3.3. Predicted binding sites for TFs in the *AGT* core promoter. Sites were predicted using the BioBase TRANSFAC and ALGGEN PROMO databases. Green underlined letters represent SNPs at -20, -18, and -6 respectively from left to right. The A between two lines represent the initiation start site. Please see **Tables S1-S4** in the **Appendix** for information about the TFs (including abbreviation and function).

***Angiotensinogen* SNPs in Organ Fibrosis.**

The severity of fibrotic diseases in the heart and kidney is influenced by ANGII. In the heart, ANGII plays a role in the hypertrophy of cardiomyocytes, fibroblast hyperplasia, and interstitial cardiac fibrosis.[31] Similar effects are also seen in the liver. The progression of congenital hepatic fibrosis is mediated through the increase of ACE, ANGII, and TGF-β_1.[32] This was also reflected in the disease progression of liver injury following chronic hepatitis C infection, in which liver fibrosis severity was highly associated with genotypes leading to higher expression of TGF-β_1 and AGT.[33] Local expression of AGT is a requirement for the experimental induction of lung fibrogenesis;[2] additionally, ANGII is a mediator of the fibro-proliferative response in acute lung injury.[34]

In fibrosis of organs other than the lungs, inter-individual variability in the progression or severity of fibrotic disease is correlated with genetic variants in *AGT* at the -20, -18, and -6 positions. In a Spanish IPF cohort, it was demonstrated that the AA genotype of the G-6A SNP was significantly associated with disease progression as measured by changes in the alveolar-arterial oxygen gradient over time.[35] This same genotype was also linked to an increase in hepatic fibrosis in people with chronic hepatitis C infections and in advanced liver fibrosis in the severely obese.[33, 36] In the heart, the G-6A and A-20C SNPs are associated with an increase in mean carotid intimal-medial thickening in females.[37] In a similar manner, both of these SNPs displayed a significant relationship in liver cirrhosis in patients with chronic hepatitis B.[38] The G-6A SNP is found in partial linkage disequilibrium with A-20C and C-18T. As a consequence, a

higher frequency of the A allele at -6 is found with C alleles at the −20 and −18 positions. Due to the scarcity of the C-18T genotype in the human population, G-6A is more frequently seen with A-20C.

The progression of fibrosis is exacerbated when variants in *AGT* are inherited in conjunction with variants in other genes. For example, the inheritance of variants in *TGF-β_1* and *AGT* together is associated with increased staging of hepatic fibrosis.[33] Individually, these variants are correlated with higher stages of fibrosis; however, the inheritance of both the Arginine/Arginine genotype in codon 25 of *TGF-β_1* and the AA genotype in −6 of *AGT*, together, led to more progressive fibrosis than either variant alone [ibid]. This effect was also associated with advanced hepatic fibrosis in obese patients with NAFLD.[36] The interaction of G-6A of *AGT* with the insertion allele of *ACE* led to an increase in the mean IMT in the population as a whole.[37]

Manipulation of the ANG System Attenuates Fibrosis.

Two critical events that are involved in the abnormal wound healing response are the apoptosis of AECs and the accumulation of collagen. In bleomycin-induced models of pulmonary fibrosis, intratracheal administration of bleomycin in the presence of Losartan, an AT_1 selective-ARB, reduced markers of apoptosis and collagen accumulation in mice. Apoptosis of AECs were detected using *in situ* end labeling of fragmented DNA and by immunohistochemistry for the active form of caspase-3 that were co-localized to MNF-116, a

marker for type II AECs.[9] Reductions in collagen accumulation were observed on histological sections of mice lungs and by quantitative measures of hydroxyproline content.[9] Histologically, these lung sections from mice treated with Losartan appeared grossly similarly to control mice.[9] Similar findings were also observed in amiodarone-induced lung fibrosis in rat models.[39]

Conclusions.

The local ANG system within the lung are required for experimental lung fibrosis. In IPF, there is an up-regulation in AGT mRNA and protein, its effector peptide, ANGII, and cathepsin D along with the down-regulation in ACE-2.[1, 7-8, 11, 40] All of these events favor the production of the profibrotic peptide, ANGII, providing support for the role of the ANG system in pulmonary fibrosis. Studies in the liver demonstrated that *AGT* transcription can be influenced by SNPs located at the -20, -18, and -6 positions. Additionally, in human pulmonary fibroblasts, TGF-β_1 can also regulate the transcription of *AGT.* From this, I hypothesize that the presence of these SNPs in pulmonary fibroblasts can also influence the rate of *AGT* transcription in the presence or absence of TGF-β_1 thereby promoting the fibrotic response. In addition to altering *AGT* transcription, these SNPs are associated with the severity and progression of various diseases - which are also predicted to be associated with the severity of IPF (please see details in **Chapter 4**).

A Preliminary Investigation on the Effects of Promoter SNPs in Regulating *AGT* Transcription in Pulmonary Fibroblasts

Introduction.

Activated epithelial cells and myofibroblasts are sources of ANGII in the lung. The profibrotic effect of ANGII is mediated through the stimulation of TGF-β_1 and the induction of EMT or fibroblasts into myofibroblasts. Excess deposition of collagen by myofibroblasts leads to the final result of fibrosis. Recent work showed that AGT mRNA and protein are constitutively expressed in human lung myofibroblasts in response to "autocrine loops" of TGF-β_1 and AGT expression which drive and propagate each other unless interrupted.[2] The up-regulation of *AGT* by TGF-β_1 is mediated through the *AGT* core promoter, which contains SNPs, A-20C, C-18T, and G-6A.[28-30]

SNPs located at the -20, -18, and -6 positions in the *AGT* core promoter alter its transcriptional rate in non-pulmonary cells. In hepatocytes, the presence of the CC haplotype at -20 and -18 respectively, resulted in more than a two-fold increase in transcription rate when compared to the AT haplotype.[29] Similarly, the A allele at -6 had a higher transcription rate in *AGT* than the G allele.[30] The effects of these SNPs in pulmonary cells have yet to be studied. Therefore, it is important to see if the effects of these SNPs in pulmonary cells parallel those seen in hepatocytes.

These same SNPs have been associated with the severity and/or progression of various diseases. Our lab, in collaboration with Spanish researchers, utilized a Spanish cohort to

demonstrate that the AA genotype of G-6A was significantly associated with disease progression in IPF as measured by the alveolar-arterial oxygen gradient over time.[35] A recent study by our lab also demonstrated that *AGT* variants at the -20 and -6 positions predicted low diffusing capacity among cohorts in the United States and Spain (details in **Chapter 4**). Studying the effects of SNPs in *AGT* in IPF can serve as a gateway to study fibrotic diseases in other organ systems. These SNPs have the potential to be disease biomarkers**.** Identifying carriers of these haplotypes in *AGT* will enable the identification of an IPF sub-population that may show greater responsiveness to treatment with angiotensin receptor blockers (ARBs) – thus helping to personalize treatment.

Materials and Methods.

Cell Culture. IMR-90s, a human fetal lung fibroblast cell line [American Type Culture Collection (ATCC), Manassas, VA) were cultured on collagen I-coated plates in Eagle's Modified Minimal Essential Media [(MEM) Sigma Aldrich, St. Louis, MO] supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (to yield complete MEM media) until they were ready for treatment. Prior to treatment, cells were washed three times with serum-free MEM media and exposed to 24 hours of serum-starvation. Porcine TGF-β_1 treatment (R&D Systems, Minneapolis, MN) at a final concentration of 2 ng/mL was used on IMR-90s (as optimized previously by Abdul-Hafez et al).[41] Additionally, experiments utilized IMR-90s that were at $\leq$ 15 in passage number.

RNA Isolation and Real-Time RT-PCR. IMR-90s ± TGF-β_1 treatment were harvested on ice from 6-well collagen-coated plates using 1 mL of Trizol Reagent (Invitrogen, Carlsbad, CA) per well. RNA was extracted according to the manufacturer's protocol. From 1 μg of total RNA, first strand cDNA synthesis was performed using the following reagents: dNTPs, Superscript II Reverse Transcriptase, oligo dT_{12-18}, 5x First Strand Buffer, DTT, and RNaseOUT. This was followed by real-time RT-PCR using 50 ng cDNA synthesized from the total RNA with the SYBR Green PCR Kit (Applied Biosystems, Foster City, CA) and 0.2 μM of primers for human *AGT*: - forward: 5'-GAG CAA TGA CCG CAT CAG-3' and reverse: 5'-CAC AGC AAA CAG GAA TGG-3' - and human *β-actin* - forward: 5'-AGG CCA ACC GCG AGA AGA TGA CC-3' and reverse: 5'-GAA GTC CAG GGC GAC GTA GC-3'. Each sample was subjected to the following conditions: 95°C for 10 minutes followed by 95°C for 30 seconds, 55°C for 37 seconds and 72°C for 37 seconds for 40 cycles terminating with the dissociation curve analysis (95°C for 1 minute, 55°C for 30 seconds, and 95°C for 30 seconds). The comparative CT method (fold-change = $2^{-\triangle\triangle CT}$; $\triangle CT = CT_{AGT} - CT_{\beta\text{-}ACTIN}$; $\triangle\triangle CT = \triangle CT_{TREATMENT} - \triangle CT_{CONTROL}$) was used to obtain the relative fold change in *AGT* expression (normalized to *β-actin*).

p0LUC-AGT Reporter Assay. The p0LUC-AGT reporter plasmid generated by Dr. Alan Brasier was sequenced to determine the *"AGT* haplotype" it contained (see **Supplementary Figure S1** in **Appendix**). Primer walking was utilized to determine the complete sequence of the plasmid before proceeding to site-directed mutagenesis. Site-directed mutagenesis was used to generate the ATG and CTA "haplotype" at -20, -18, and -6 respectively (GenScript, Piscataway,

NJ). "Mutated" p0LUC-AGT were verified through sequencing at the Research Technology Support Facility (RTSF) at Michigan State University (see **Supplementary Figure S2** in **Appendix**). IMR-90s were co-transfected with p0LUC-AGT and pRL-CMV using Fugene6 (Promega, Madison, WI) at an optimal ratio of 1 µg: 6 µL. After 4 hours of transfection, the transfection solution was removed and replaced with new media $\pm$ TGF-β_1 at a final concentration of 2 ng/mL. After 24 hours of treatment, cells were harvested in lysis buffer and assayed using the Dual Luciferase Reporter Kit (Promega, Madison, WI) on a TD-20/20 Luminometer (Turner Designs) according to the manufacturer's protocol.

Transcription Factor Complex Pull-Down. Single-stranded oligonucleotides containing the core promoter from -46 to +22 with the CTA or ATG haplotype (were generated with a 5'-biotinylated modification [(Integrated DNA Technologies, Coralville, IA) **Table 3.1** for sequences]. The -18 SNP was unaltered to reflect what is in the population - our previous study demonstrated a lack of diversity at this position in both cohorts from the United States and Spain. Respective antisense and sense strands were allowed to anneal to generate double-stranded biotinylated oligonucleotides containing the CTA or ATG haplotype. These double-stranded oligonucleotides were immobilized to streptavidin magnetic beads (Promega, Coralville, IA). 3.75 µg of charged streptavidin beads were bound to 75 µg of nuclear extracts harvested from IMR-90s $\pm$ TGF-β_1. Nuclear extracts were harvested using the protocol published by Wu from *Methods in Molecular Biology* with minor revisions.[42] Briefly, cells were gently washed in 1x PBS containing EDTA-free protease inhibitor cocktail (PBSi). Cells were then

scraped in 1x PBSi and centrifuge at 4°C at 550g for 5 minutes to obtain a cell pellet. The solution was decanted and replaced with 200 µL of Buffer A (10 mM HEPES at pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM sucrose, 0.5% NP-40, EDTA-free protease inhibitor cocktail, and phosphatase inhibitor cocktail). Cells were gently mixed by flicking the tube and setting it on ice for 10 minutes with brief vortexing at 2 minute intervals. After 10 minutes on ice, cells were centrifuge at 2600g for 30 seconds. The supernatant in this tube is the cytoplasmic fraction and was transferred to a new tube and stored at -80°C for future use. The remaining cell pellet was resuspended in 150 µL of Buffer B (20 mM HEPES at pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 2.5% glycerol, EDTA-free protease inhibitor cocktail, and phosphatase inhibitor cocktail) and subjected to 3-4 freeze-thaw cycles before quantitation of protein concentration. These nuclear fractions were aliquoted and stored at -80°C until further use.

Table 3.1. Sequences used for the generation of double-stranded biotinylated AGT oligonucleotides containing the ATG or CTA haplotype at -20, -18, and -6 respectively.

PRIMER NAME	SEQUENCE
ATG_FORWARD	5'-Biotin-CCA CCC CTC AGC TAT AAA TAG GGC ATT GTG ACC CGG CCG GGG GAA GAA GCT GCC GTT GTT CT-3'
ATG_REVERSE	5'-AGA ACA ACG GCA GCT TCT TCC CCC GGC CGG GTC ACA ATG CCC TAT TTA TAG CTG AGG GGT GG-3'
CTA_FORWARD	5'-Biotin-CCA CCC CTC AGC TAT AAA TAG GGC CTT GTG ACC CGG CCA GGG GAA GAA GCT GCC GTT GTT CT-3'
CTA_REVERSE	5'-AGA ACA ACG GCA GCT TCT TCC CCT GGC CGG GTC ACA AGG CCC TAT TTA TAG CTG AGG GGT GG-3'

Panomics DNA/TF Array. Nuclear extracts from IMR-90s treated with TGF-β_1 and pulled-down with the biotinylated-CTA or -ATG streptavidin beads were used as the starting material for the Panomics TF/DNA Array I and II as followed by the manufacturer's protocol. TGF-β_1 treated cells were utilized in this assay as it was the only condition that yielded a noticeable difference in p0LUC-AGT activity with the different *AGT* haplotypes. Briefly, the starting material was generated in a similar manner described in the ***Transcription Factor Complex Pull-Down*** section. After the pull-down, proteins were dislodged from the oligonucleotides by incubating the samples at 4°C for 30 minutes in 2 M NaCl. After incubation, the solution containing the TFs was separated from the streptavidin-bound oligonucleotides with a magnet. A 1:1 mixture of pre-labeled 5'-biotinylated-TF probes (contained in the Panomics TF/DNA Array Kits) were incubated with this solution containing 25 µg of protein that were bound to the *AGT* core promoter. The DNA/protein complexes were extracted from a 2% agarose gel and eluted with the supplemented buffers in the kit as a means to separate the bound probes from the free ones. 10 µg of the eluted DNA/protein complexes were denatured at 95°C for 3 minutes with a quick chill at 4°C for 2 minutes to liberate the probes from the TFs. This solution was then added into a hybridization chamber containing the activated membrane (activation occurred at 42°C for 2 hours in the supplemented Panomics Pre-hybridization Buffer) to allow the free probes to bind the complementary oligonucleotides "spotted" on the

membrane in duplicates. Hybridization occurred at 42°C for 20 hours and was followed by blocking at room temperature for 20 minutes with an additonal 15 minutes in blocking buffer containing streptavidin-HRP. Membranes were washed 3 times at room temperature before visualization with chemiluminescent substrate.

Preliminary Results.

***TGF-β_1 Induces AGT Transcription*.** TGF-β_1-inducible-*AGT* transcription was reproducible in a new culture of IMR-90s obtained from ATCC (**Figure 3.4**). Data throughout this thesis involving IMR-90s were generated using these set of cells. Prior data from Abdul-Hafez et al. demonstrated that this increase in *AGT* was transcriptionally regulated and not an effect of mRNA stability.[41]

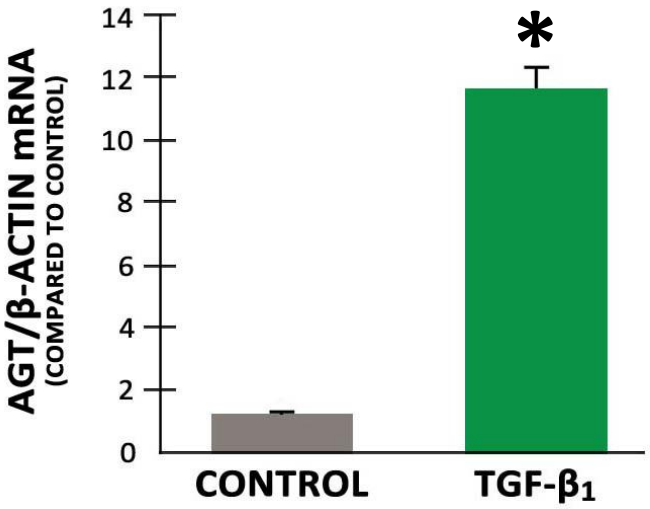

Figure 3.4. TGF-β_1 induces *AGT* transcription in IMR-90s. Data are presented as mean ± SEM with n = 4; ∗ p = 0.0079 using Mann-Whitney test.

Influence of -20 and -6 AGT Haplotype on TGF-β_1-Inducible AGT Transcription. Indirect measurements of *AGT* transcription using the p0LUC-AGT reporter construct containing the CTA or ATG haplotype suggested that TGF-β_1-inducible-*AGT* transcription was altered with promoter variants. The "CTA haplotype" showed an increase trend in reporter activity compared to the "ATG haplotype." Additionally, this difference was more sensitively observed with TGF-β_1 stimulation (**Figure 3.5B**) - at baseline, the difference in *AGT* transcription between the CTA and ATG haplotypes was less noticeable (**Figure 3.5A**).

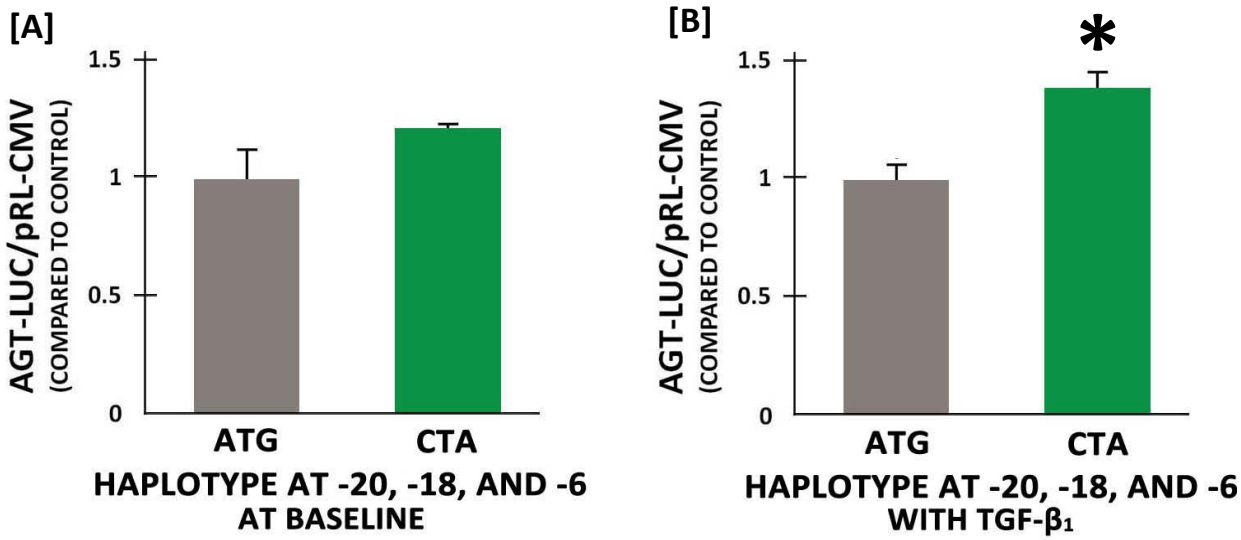

Figure 3.5. Effects of AGT haplotypes on *AGT* transcription. [A] At baseline, the SNPs at the -20 and -6 positions in the core promoter of *AGT* did not influence its transcription rate as measured by the Dual Luciferase Assay. **[B]** However, the addition of TGF-β_1 resulted in significant differences between transcription rate with the ATG and CTA haplotypes. Data are presented as mean $\pm$ SEM.

Influence of -20 and -6 AGT Haplotype on Transcription Factors. Screening of TFs that could be altered with variants at the -20 and -6 positions in *AGT* was performed with TF/DNA Arrays I and II from Panomics. From this, a variety of differences in TF binding affinity to the ATG or CTA variants were observed in this first experiment (**Figure 3.6**). The predicted binding sites for Upstream Stimulatory Factor (USF-1), Activation Protein (AP-2), and Nuclear Factor Erythroid-Derived (NF-E2) overlaps the -20 and -18 SNPs (**Figure 3.6A**). Even though the binding site for the cAMP Response Element Binding Protein (CREB-BP1) is present down-stream from the -20 and -6 positions, a large effect was observed (**Figure 3.6B**) between the variants. Additionally, differences in TFs that had no predicted binding sites on the core promoter of *AGT* were also observed (**Figure 3.6C**). Greater binding of the TF is reflected in the darker density of the dots observed after chemiluminiscent detection. It should be noted that the side-by-side dots depicted in **Figure 3.6** represent technical replicates from the same sample and not from independent ones. Nuclear extracts from TGF-β_1 treated IMR-90s were given priority in this assay as this was the condition that showed greater sensitivity towards a difference in p0LUC-AGT reporter activity with the two *AGT* haplotypes.

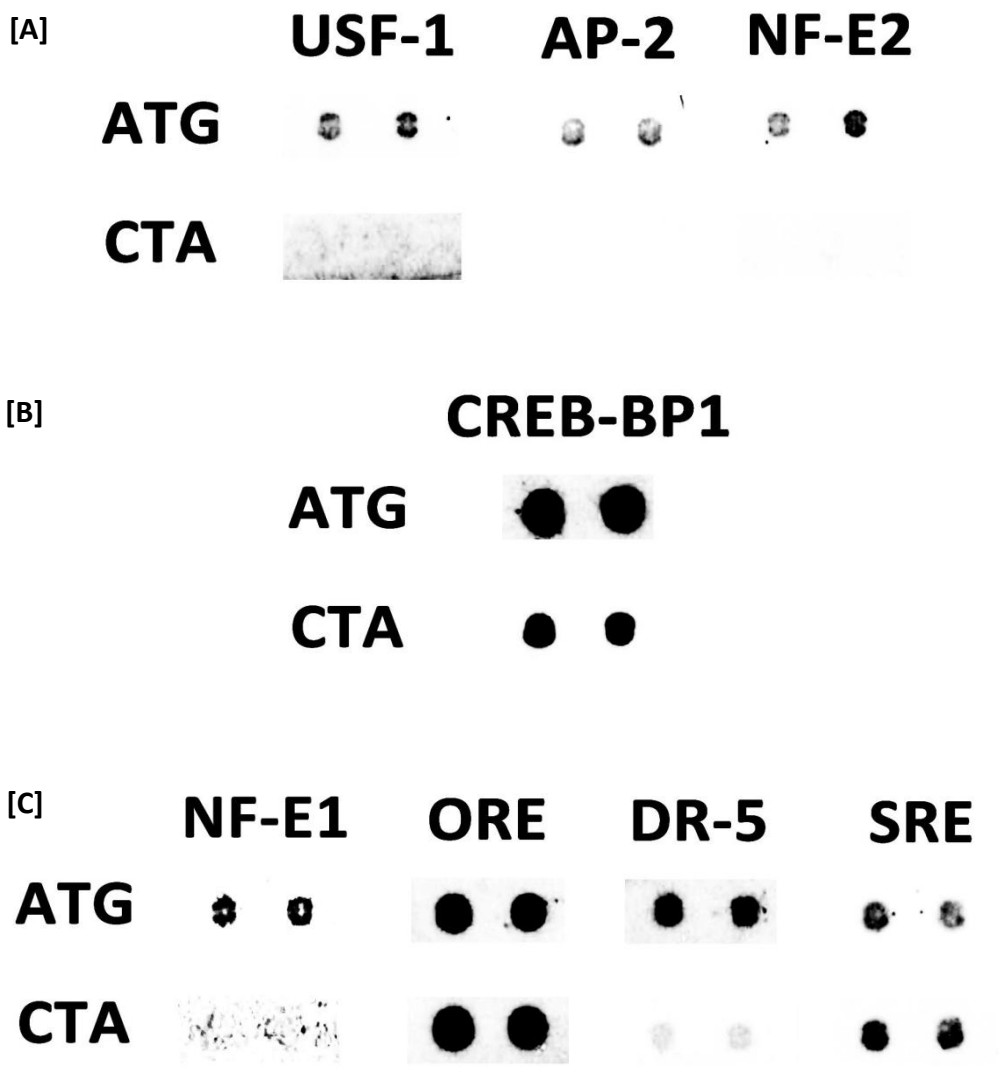

Figure 3.6. Alterations of TF binding with *AGT* haplotypes at -20, -18, and -6. Effects of *AGT* haplotypes on TFs with **[A]** predicted binding sites overlapping the -20 and/or -18 SNP, **[B]** predicted binding sites down-stream from the SNPs of interest, and **[C]** no predicted binding sites in the core promoter. Each dot represents technical duplicates for the listed TF. USF-1 = upstream stimulatory factor-1; AP = activation protein-2; NF-E2 = nuclear factor erythroid-derived-2; CREB-BP1 = cAMP response element binding protein; NF-E1 = YY1 = nuclear factor erythroid-1; ORE = osmotic response element; DR-5 = TNF receptor superfamily member 10; SRE = sterol regulatory element.

Discussion.

Alteration of AGT Transcription with Promoter SNPs. In hepatocytes, higher rates of *AGT* transcription were observed with the presence of the CC haplotype at -20 and -18 and the A allele at -6.[29-30] Preliminary results indicate a similar trend in human pulmonary fibroblasts, where the CTA haplotype at -20, -18, and -6 had a greater increase in *AGT* transcription compared to the ATG haplotype. However, in order to validate this observation, the Dual Luciferase Reporter assay has to be repeated with multiple plasmid preparations in multiple experiments with further optimization in order to see a significant difference in activity.
In this research, we did not carry out studies on the -18 position due to the lack of diversity of this SNP in the population (that we observed in cohorts from the United States and Spain in our prior study).[43] The higher rates of *AGT* transcription are predicted to generate higher levels of the profibrotic peptide, ANGII. Higher levels of ANGII would in turn lead to greater severity in pulmonary fibrosis. In a prior study, our laboratory discovered that the CC genotype at -20 or the AA genotype at -6 predicted lower measures of diffusing capacity in cohorts of IPF from the United States and Spain.[43] The finding that these SNPs influence the rate of *AGT* transcription provides further support for a role of the ANG system in IPF and a biological significance of these variants as potential biomarkers.

Unlike hepatocytes, the difference in transcription rate with the *AGT* haplotypes were more sensitively observed with TGF-β_1 stimulation in pulmonary fibroblasts. TGF-β_1 can stimulate the transition of fibroblasts into myofibroblasts. In excess of TGF-β_1, myofibroblasts

can accumulate to form myofibroblastic foci. Currently, the number of these myofibroblastic foci is the only histopathological predictor of mortality in IPF patients.[44] In myofibroblasts, there is an autocrine ANGII-TGF-β_1 loop where TGF-β_1 induces *AGT* transcription to generate the effector peptide, ANGII.[2] ANGII can stimulate TGF-β_1 synthesis and TGF-β_1 can induce *AGT* transcription and myofibroblast formation in both an autocrine and paracrine manner. This never-ending cycle perpetuates the accumulation of myofibroblasts and extracellular matrix proteins along with the apoptosis of nearby AECs induced by ANGII. These events contribute to the abnormal wound healing underlying IPF.

Effects of AGT Haplotypes on the Binding of TFs. Preliminary data suggests that the presence of the -20 and -6 SNPs in the core promoter of *AGT* was able to alter the binding affinities of several TFs. The changes in these TFs can provide insight into the regulation of TGF-β_1-inducible *AGT* transcription. Earlier work from our laboratory demonstrated that JunD (an AP-1 TF) and HIF-1α mediated TGF-β_1-inducible *AGT* transcription.[41] In that published study, the CCA *AGT* "haplotype" at the -20, -18, and -6 positions, was contained in the reporter constructs. This haplotype is predicted to yield the highest rate of *AGT* transcription. In our preliminary study, the CCA haplotype was not studied due to the lack of diversity at the -18 position in the human population. It will be interesting to see if the -18 SNP (CTA vs CCA) changes the requirement of HIF-1α and JunD in mediating the transcription of *AGT.* In a similar manner, the -20 and -6 SNPs may also alter HIF-1α and JunD mediated TGF-β_1-inducible *AGT* transcription.

The recognition site for HIF-1 can also be recognized by CRE binding factors, ATF-1 and CREB-BP1, the latter of which showed increased binding to the ATG haplotype (**Figure 3.6B**).[45-47] It is hypothesized that the increased affinity for CREB-BP1 with the ATG haplotype may outcompete HIF-1α, thereby limiting *AGT* transcription (**Figure 3.7**). Currently, our lab is investigating the effects of these *AGT* haplotypes on the binding of JunD and HIF-1α. TFs that had no predicted binding sites to the promoter were also observed to change with these variants. This suggests additional interactions between TFs themselves along with their binding sites. It is hypothesized that these TFs interact with TFs bound to the promoter with repressor-like qualities to down-regulate *AGT* transcription with the ATG haplotype that showed lower transcription rate (**Figure 3.5B and 3.6C**)]. Further experiments are required to parse out the effects of these TFs.

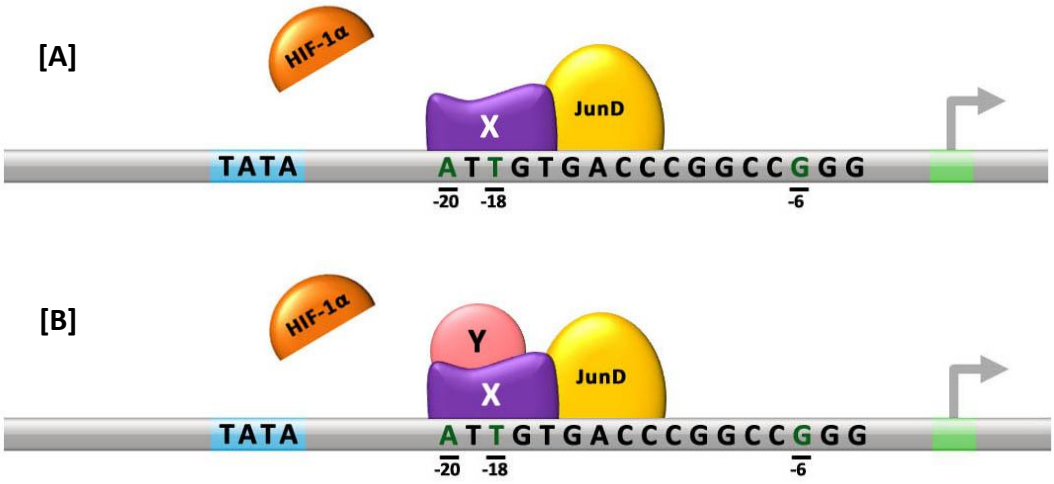

Figure 3.7. Predicted effects of the ATG *AGT* haplotype on the regulation of *AGT* transcription. [A] "Protein X" competes with HIF-1α due to similar binding sites to the hypoxia response element. **[B]** "Protein X" may also interact with "Protein Y" to down-regulate *AGT* transcription. "Protein X" may represent USF-1, AP-2, or CREB-BP1 or a combination of these as an inhibitory complex. Candidates for "Protein Y" includes those mentioned for "Protein X" and also NF-E1 and DR-5. HIF-1α = hypoxia inducible factor-1.

In hepatocytes, *AGT* transcription is regulated in part by the binding of the AGT Core Promoter Element Binding Factor (AGCF-1) to the AGT Core Promoter Element (AGCE-1).[29] This interaction is species-specific and is influenced by molecular variants in *AGT* located at the -20 and -18 positions.[29] The AT *AGT* variant at -20 and -18 positions respectively, had 40% of the transcriptional activity observed with the CC *AGT* variant.[29] Part of the AGCF-1 complex consists of USF-1 and the presence of the C allele at -20 favors its binding to the AGCE-1 in hepatocytes.[29, 48] In our preliminary study in pulmonary fibroblasts, the opposite was observed where it appeared that USF-1 favored the A allele at -20. This discrepancy may reflect the cell specificity of *AGT* regulation that may be contributed in part by the presence of a co-activator in the AGCF-1 complex in hepatocytes and not in pulmonary fibroblasts. In pulmonary fibroblasts, the role of AGCF-1 in *AGT* transcription has not been elucidated and would be interesting to test in future studies. Qyang et al. demonstrated that the presence of USF DNA-binding activity is not sufficient to suggest USF function in transcriptional activation, as in some cells, the activation domain of USF may be masked.[49] Therefore, in pulmonary fibroblasts, USF may be rendered inactive due to the presence of a co-repressor masking the activation domain. Another explanation for this discrepancy is that the predicted binding sites for USF and HIF overlaps each-other and may result in competition for that site (**Figure 3.3** and **Figure 3.7**). The alteration of the binding sites with the -20 SNP may increase the odds of one TF over the other by increasing the affinity for that TF to the site. The Panomics DNA/TF Array contains complementary oligonucleotides for the binding sites of TFs. The DNA-binding specificities of

USF-1 and USF-2 are identical,[49] therefore the differentiation between the binding of USF-1 or USF-2 to these spotted oligonucleotides on the Panomics membrane may not be sensitive enough to distinguish between these two TFs. In this case, the increased intensity of the spot may also represent a preference of USF-2 for the A allele at -20. In the vicinity of hypoxia response elements, USF-2 can act as a repressor for gene transcription.[50]

Limitations and Future Studies.

Indirect Measures of AGT Transcription. It is important to note that these preliminary data require further validation studies but still provide clues into the transcriptional regulation of *AGT* in pulmonary fibroblasts. In this study, indirect measures of *AGT* transcription were performed using the Dual Luciferase Reporter assay, which is a widely used method in cell biology research to study transcription rates. However, a limitation of this method is that it does not directly measure endogenous *AGT* transcription, which can best be observed using nuclear run-on assays. To accurately study the effects of *AGT* haplotypes on its transcription would require two human pulmonary fibroblast cell lines that inherently contain only the ATG or CTA *AGT* haplotype at the -20, -18, and -6 positions. Although this is the ideal condition to use, in reality obtaining two fibroblast cell lines with nearly identical genetic background except for the *AGT* haplotypes will be difficult. A less stringent alternative is to use fibroblasts with an identical *AGT* sequence that only alter at the -20 and -6 positions as other SNPs in *AGT* can also affect is transcription rate (such as M235T). Real-time RT-PCR can also be utilized with these

nearly identical cells to help determine if steady state *AGT* levels are affected with these two *AGT* haplotypes. Nuclear extracts obtained from either of these cells would provide a more accurate starting material for future experiments since the interaction of TFs with the haplotypes of interest are naturally occurring as opposed to the experiments reported here where synthetic oligonucleotides were used to study these interactions.

DNA/TF Interactions. Although the data from the Panomics DNA/TF Array are not sufficient by itself to demonstrate that the alterations in the binding of several TFs are influenced by these SNPs, it does provide a starting point for candidate TFs to be used in future studies. Further validation experiments can be confirmed with electrophoretic mobility shift assays (EMSAs), co-immunoprecipitation, nitrocellulose filter-binding, or by foot-printing assays. Coupling EMSAs with western blotting, mass spectroscopy, or a "super-shift" assay can help to identify the associated TF(s).[51] However, unlike nitrocellulose filter-binding assays where it is difficult to distinguish the different stoichiometry of TF/DNA complexes, EMSAs do not have this limitation.[51] Although foot-printing assays provide more direct information on the binding sites, optimization to produce a foot-print signal are more time-consuming and difficult compared to EMSAs or nitrocellulose filter-binding assays.[51] In the ideal scenario, nuclear extracts from nearly identical fibroblasts (except for the *AGT* haplotype at -20 and -6) will be used as starting materials for EMSAs using synthetic biotinylated oligonucleotides against binding sites for USF-1, AP-2, NF-E2, CREB-BP1, NF-E1, ORE, DR-5, and SRE (candidate TFs from screening with the Panomics DNA/TF array). Prior studies demonstrated that TGF-β_1-inducible

AGT transcription was mediated by HIF-1α and JunD; therefore, both of these TFs will also be included in these assays.

Protein-Protein Interactions. Co-immunoprecipitations, pull-down assays, cross-linking protein interaction analysis, or far-western blot analysis may be used to study protein-protein interaction present on the *AGT* core promoter. In these studies, candidate "baits" or target proteins will include HIF-1α, JunD, USF-1, AP-2, NF-E2, and CREB-BP1. It would be interesting to see if one or more of these proteins interact with NF-E1, ORE, DR-5, or SRE, as these TFs were observed to be altered on our Panomics screen without predicted binding sites to the *AGT* core promoter. In the preliminary streptavidin-mediated pull-down assay, there is the possibility that interacting proteins were lost, however this was not quantitated in the study. In the future, collection of the wash fractions for Western Blotting can be used to monitor the amount of protein loss. Additionally, it is unknown if these protein-protein interactions are strong, weak, or transient. Therefore, slight alterations in the binding and washing buffer conditions could have perturbed their interactions. This suggests that in future pull-down experiments, different buffer compositions should be used to find the optimal buffers to help minimize the loss of protein-protein interactions.

(Co)-Repressor and (Co)-Activator Functions. The effects of these TFs on *AGT* transcription as (co)-repressors or (co)-activators will need to be elucidated. The identities for these candidate TFs will be obtained from the preliminary data generated by the Panomics DNA/TF Arrays (**Figure 3.6**) and verified by future EMSAs. Mutations in the p0LUC-AGT reporter

construct at the predicted binding sites for the TF of interest will be generated in the background CTA or ATG *AGT* haplotype using site-directed mutagenesis and will be confirmed with sequencing. For some TFs, dominant-negative constructs may be used as alternatives. It is important to verify that these mutations do not create new binding sites in the promoter by rescreening the sequence using a TRANSFAC database. Once these mutations are created, induction of promoter activity will be measured in the presence and absence of TGF-β_1 using the Dual Luciferase Reporter Assay. Additionally, expression vectors for these candidate TFs can be co-transfected with the p0LUC-AGT construct containing the *AGT* haplotypes. If fibroblasts with endogenous CTA or ATG *AGT* haplotypes are available, real-time RT-PCR can be used to determine if over-expression of the TFs, dominant-negative constructs, or siRNAs are sufficient to induce or repress *AGT* expression. If protein-protein interactions are observed, combinations of specific knock-downs of the involved TFs using siRNAs will be utilized to help determine if both TFs are essential in regulating *AGT* transcription. The results from these future studies will provide support for the effects of *AGT* SNPs on the binding of TFs to influence the rate of *AGT* transcription.

APPENDIX

Table S1. Description of TFs with predicted binding sites to the *AGT* core promoter with a role in differentiation.*

ROLE IN DIFFERENTIATION		
TRANSCRIPTION FACTOR	**FUNCTION**	**ASSOCIATION WITH DISEASES**
EBF (EARLY B-CELL FACTOR)	Cytokine-mediated signaling, B-cell differentiation, retinogenesis, and brain development.	Hodgkin Lymphoma, Sjogren Syndrome, and arteriosclerosis.
FOXL-1 (FORKHEAD BOX-L1)	Hepatocyte differentiation and proliferation, Wnt signaling, and Peyer's Patch development.	Low-grade Fibromyxoid Sarcoma
GABP-α (GA BINDING PROTEIN TF ALPHA SUBUNIT OF 60 kDa)	Regulates myeloid progenitor cell differentiation.	Hepatocellular Carcinoma
PAX-3 (PAIRED BOX-3)	Melanocyte differentiation, melanogenesis, skin and eye pigmentation, neurogenesis, and organ development.	Pigmented Nevus and Waardenburg Syndrome.
PPAR-γ (PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA)	Promotes adipocyte differentiation.	Psoriasis, HIV infections, Breast Cancer, and Type II Diabetes
SPI-B	B-cell receptor signaling, myeloid cell differentiation, immunity, apoptosis, and dendritic cell development.	B-cell lymphoma and Biliary Cirrhosis
STAT-4 (SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 4)	Cytokine-mediated signaling, IL-4 production, and TH2 cell differentiation.	Rheumatoid Arthritis, psoriasis, and Sjogren Syndrome.
USF-1 (UPSTREAM STIMULATORY FACTOR-1)	cAMP synthesis, inhibits G2-M transition and trophoblast differentiation.	Type II diabetes, dyslipidemia, and coronary atherosclerosis.

*Organization of Supplementary Tables S1 - S4 is not meant to indicate the importance of one function over another.

Table S2. Description of TFs with predicted binding sites to the *AGT* core promoter involved in signaling pathways.

WNT AND OTHER SIGNALING PATHWAYS		
TRANSCRIPTION FACTOR	**FUNCTION**	**ASSOCIATION WITH DISEASES**
ATF-3 (ACTIVATING TF-3)	Wnt signaling, cytokine production, cell proliferation, and apoptosis.	Arteriosclerosis, Diabetes, Leiomyoma, and Skin Cancer.
CDX-2 (CAUDAL TYPE HOMEOBOX-2)	Wnt signaling and trophectodermal cell differentiation.	AML, colorectal and ovarian cancer, intestinal neuroendocrine tumors
ELK-1 (EST ONCOGENE MEMBER)	MAPKKK, EGFR, GPCR, and Ras signaling pathways, skeletal muscle tissue development, and detection of light stimulus, inhibits neuron apoptosis and promotes cell proliferation.	
JUN (JUN ONCOGENE)	EGFR signaling, anti-apoptosis, and RNA processing.	Non-small Cell Lung Cancer, Prostate Cancer, Myeloid Leukemia, Retinitis Pigmentosa, Glomerulonephritis, and Psoriasis.
NR1-B2 (RETINOIC ACID RECEPTOR BETA)	Regulates retinoic acid receptor signaling, induction of apoptosis, axonogenesis, synaptic plasticity, and axon regeneration.	Breast and Cervical Cancer.
PR (PROGESTERONE RECEPTOR)	APK activation, Wnt receptor signaling, cell adhesion, ovulation, embryo implantation, and parturition.	Obesity, Breast and Ovarian cancers.
SOX-17 (SRY SEX DETERMINING REGION Y-BOX)	Canonical Wnt signaling and cell fate determination.	Breast and Gastric Cancers.

AML = acute myeloid leukemia.

Table S3. Description of TFs with predicted binding sites to the *AGT* core promoter with a role as activators or co-repressors.

ACTIVATORS AND CO-REPRESSORS		
TRANSCRIPTION FACTOR	**FUNCTION**	**ASSOCIATION WITH DISEASES**
CEBP (CCAAT-ENHANCER BINDING PROTEIN)	Transcriptional co-activator that acts in lung development and cell differentiation.	Hepatocellular Carcinoma, Myeloid Leukemia and Myelodysplastic Syndromes.
ETS-1 (HOMOLOG 1 OF V-ETS ERYTHROBLASTOSIS VIRUS E26 ONCOGENE)	Transcriptional activator that acts in apoptosis, angiogenesis, decidualization, heart development, and cytokine secretion.	Arthritis and Ovarian Cancer
HES-1 (HAIRY AND ENHANCER OF SPLIT 1)	Co-repressor in Notch signaling, neurogenesis, organ development, and bone resorption.	Meningioma, Crohn's, Down Syndrome, and Osteosarcoma
KID-3	Co-repressor in osteogenic differentiation, member of the KRAB box family	
NF-E2 (NUCLEAR FACTOR ERYTHROID DERIVED-2)	Co-activator in erythrocyte development, platelet formation, and blood coagulation, regulates megakaryocyte differentiation.	Thrombocytopenia and Polycythemia Vera
USF-2 (UPSTREAM STIMULATORY FACTOR-2)	Co-activator in rRNA transcription, central nervous system development, and lactose biosynthesis.	Hydronephrosis

Table S4. Description of TFs with predicted binding sites to the *AGT* core promoter.

OTHER ROLES		
TRANSCRIPTION FACTOR	**FUNCTION**	**ASSOCIATION WITH DISEASES**
AP-2 (ACTIVATION PROTEIN-2)	Organ development, neuron migration, apoptosis, and sensory perception of sound.	Dysplastic Nevus Syndrome and Skin Cancer
ER (ESTROGEN RECEPTOR-1)	Sexual and reproductive processes.	Alzheimer's, Parkinson's, atherosclerosis, PCOS, Breast Cancer, and osteoporosis
FOXP-3 (FORKHEAD BOX-P3)	T-cell activation.	Multiple Sclerosis, Diabetes, Leukemia, Crohn's, and Breast Cancer
HIF-1α (HYPOXIA INDUCIBLE FACTOR 1-ALPHA)	Apoptosis and angiogenesis.	Keloids, Huntington's, Breast and Lung Cancer, Altitude Sickness and Heart Disease
MEF-2 (MYOCYTE ENHANCER FACTOR-2)	Mitochondrial organization, cardiac myofibril assembly, and synaptic plasticity.	Hepatic Cancer, arteriosclerosis, and Myocardial Infarction
NR3C-1 (GLUCOCORTICOID RECEPTOR)	Inflammatory response.	Cushing Syndrome, Crohn's, Alzheimer's, schizophrenia, and Lymphocytic Leukemia
P53 (TUMOR PROTEIN 53)	Cell cycle arrest, apoptosis, senescence, DNA repair, and keratinocyte differentiation.	Breast Cancer and Li-Fraumeni Syndrome
PAX-2 (PAIRED BOX-2)	Cell proliferation and anti-apoptosis, involved in brain, eye, ear, and prostate development.	Kidney and Ovarian Cancers and coloboma.
PAX-5 (PAIRED BOX-5)	Cell cycle, apoptosis, ossification, brain development, and immunity.	Macroglobulinemia, B-cell Lymphoma, and Breast Cancer.
SALL-2 (SAL-LIKE-2)	Binds BAX promoter and the p75 neurotrophin receptor (NGFR)	
SATB-1 (SPECIAL AT-RICH SEQUENCE BINDING PROTEIN-1)	Chromatin modification, nuclear matrix organization, cytokine production, T-cell development, and cytolysis.	Glioma and Prostate Cancer.

PCOS = polycystic ovarian syndrome

GGATCCTGGGTAATTTCATGTCTGCCATCGTGGATATGCCGTGGCTCCTTGAACCTGCTTGTGTTGAAGCAG
GATCTTCCTTCCTGTCCCTTCAGTGCCCTAATACCATGTATTTAAGGCTGGACACATCACCACTCCCAACCTG
CCTCACCCACTGCGTCACTTGTGATCACTGGCTTCTGGCGACTCTCACCAAGGTCTCTGTCATGCCCTGTTAT
AATGACTACAAAAGCAAGTCTTACCTATAGGAAAATAAGAATTATAACCCTTTTACTGGTCATGTGAAACTT
ACCATTTGCAATTTGTACAGCATAAACACAGAACAGCACATCTTTCAATGCCTGCATCCTGAAGGCATTTTG
TTTGTGTCTTTCAATCTGGCTGTGCTATTGTTGGTGTTTAACAGTCTCCCCAGCTACACTGGAAACTTCCAGA
AGGCACTTTTCACTTGCTTGTGTGTTTTCCCCAGTGTCTATTAGAGGCCTTTGCACAGGGTAGGCTCTTTGGA
GCAGCTGAAGGTCACACATCCCATGAGCGGGCAGCAGGGTCAGAAGTGGCCCCCGTGTTGCCTAAGCAAG
ACTCTCCCCTGCCCTCTGCCCTCTGCACCTCCGGCCTGCATGTCCCTGTGGCCTCTTGGGGGTACATCTCCCG
GGGCTGGGTCAGAAGGCCTGGGTGGTTGGCCTCAGGCTGTCACACACCTAGGGAGATGCTCCCGTTTCTG
GGAACCTTGGCCCCGACTCCTGCAAACTTCGGTAAATGTGTAACTCGACCCTGCACCGGCTCACTCTGTTCA
GCAGTGAAACTCTGCATCGATCACTAAGACTTCCTGGAAGAGGTCCCAGCGTGAGTGTCGCTTCTGGCATC
TGTCCTTCTGGCCAGCCTGTGGTCTGGCCAAGTGATGTAACCCTCCTCTCCAGCCTGTGCACAGGCAGCCTG
GGAACAGCTCCATCCCCACCCCTCAGCTATAAATAGGGCCTCGTGACCCGGCCAGGGGAAGAAGCTGCCGT
TGTTCTAAGCTTGGCATTCCGGTACTGTTGGTAAAATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGC
GCCATTCTATCCTCTAGAGGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGATACGCCCTG
GTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGAACATCACGTACGCGGAATACTTCGAAAT
GTCCGTTCGGTTGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCACAGAATCGTCGTATGCAGT
GAAAACTCTCTTCAATTCTTTATGCCGGTGTTGGGCGCGTTATTTATCGGAGTTGCAGTTGCGCCCGCGAAC
GACATTTATAATGAACGTGAATTGCTCAACAGTATGAACATTTCGCAGCCTACCGTAGTGTTTGTTTCCAAA
AAGGGGTTGCAAAAAATTTTGAACGTGCAAAAAAAATTACCAATAATCCAGAAAATTATTATCATGGATTC
TAAAACGGATTACCAGGGATTTCAGTCGATGTACACGTTCGTCACATCTCATCTACCTCCCGGTTTTAATGA
ATACGATTTTGTACCAGAGTCCTTTGATCGTGACAAAACAATTGCACTGATAATGAATTCCTCTGGATCTAC
TGGGTTACCTAAGGGTGTGGCCCTTCCGCATAGAACTGCCTGCGTCAGATTCTCGCATGCCAGAGATCCTAT
TTTTGGCAATCAAATCATTCCGGATACTGCGATTTTAAGTGTTGTTCCATTCCATCACGGTTTTGGAATGTTT
ACTACACTCGGATATTTGATATGTGGATTTCGAGTCGTCTTAATGTATAGATTTGAAGAAGAGCTGTTTTTA
CGATCCCTTCAGGATTACAAAATTCAAAGTGCGTTGCTAGTACCAACCCTATTTTCATTCTTCGCCAAAAGCA
CTCTGATTGACAAATACGATTTATCTAATTTACACGAAATTGCTTCTGGGGGCGCACCTCTTTCGAAAGAAG
TCGGGGAAGCGGTTGCAAAACGCTTCCATCTTCCAGGGATACGACAAGGATATGGGCTCACTGAGACTAC
ATCAGCTATTCTGATTACACCCGAGGGGGATGATAAACCGGGCGCGGTCGGTAAAGTTGTTCCATTTTTTG
AAGCGAAGGTTGTGGATCTGGATACCGGGAAAACGCTGGGCGTTAATCAGAGAGGCGAATTATGTGTCA
GAGGACCTATGATTATGTCCGGTTATGTAAACAATCCGGAAGCGACCAACGCCTTGATTGACAAGGATGG
ATGGCTACATTCTGGAGACATAGCTTACTGGGACGAAGACGAACACTTCTTCATAGTTGACCGCTTGAAGT
CTTTAATTAAATACAAAGGATATCAGGTGGCCCCCGCTGAATTGGAATCGATATTGTTACAACACCCCAAC
ATCTTCGACGCGGGCGTGGCAGGTCTTCCCGACGATGACGCCGGTGAACTTCCCGCCGCCGTTGTTGTTTTG
GAGCACGGAAAGACGATGACGGAAAAAGAGATCGTGGATTACGTCGCCAGTCAAGTAACAACCGCGAAA
AAGTTGCGCGGAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGGAAAACTCGACGCAAGAA
AAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGTCCAAATTGTAAAATGTAACTGTATTCAG
CGATGACGAAATTCTTAGCTATTGTAATATTATATGCAAATTGATGAATGGTAATTTTGTAATTGTGGGTCA
CTGTACTATTTTAACGAATAATAAAATCAGGTATAGGTAACTAAAAAGGAATTCGAGCTCGAATTCCGGTC
TCCCTATAGTGAGTCGTATTAATTTCGATAAGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGG
CGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCG

Figure S1. Complete sequence of p0LUC-AGT reporter plasmid before site-directed mutagenesis (5,692 bp with ampicillin resistance). green = AGT insert; pink = firefly luciferase gene; purple = SNPs; underli ne = BamHI and HindIII RE sites respectively.

Figure S1 (cont'd)

AGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAAC
ATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGC
TCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATA
AAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATAC
CTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGT
AGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTA
ACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATT
AGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAA
GAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCC
GGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAG
GATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGG
ATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCA
ATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCG
ATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTA
CCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAAC
CAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTG
TTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCA
TCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACAT
GATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCG
CAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC
TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGG
CGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCG
GGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTG
ATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAA
AGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATC
AGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGC
ACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGG
CGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCG
GAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGT
GTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATTCGA
CGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGC
CGCAAGGAATGGTGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACGC
CGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGC
GCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCTGGCTAGCG
ATGACCCTGCTGATTGGTTCGCTGACCATTTCCGGGTGCGGGACGGCGTTACCAGAAACTCAGAAGGTTCG
TCCAACCAAACCGACTCTGACGGCAGTTTACGAGAGAGATGATAGGGTCTGCTTCAGTAAGCCAGATGCTA
CACAATTAGGCTTGTACATATTGTCGTTAGAACGCGGCTACAATTAATACATAACCTTATGTATCATACACA
TACGATTTAGGTGACACTATAGAATACAAGCTAGCTTGCATGCCTGCAGGTCGACTCTAGA

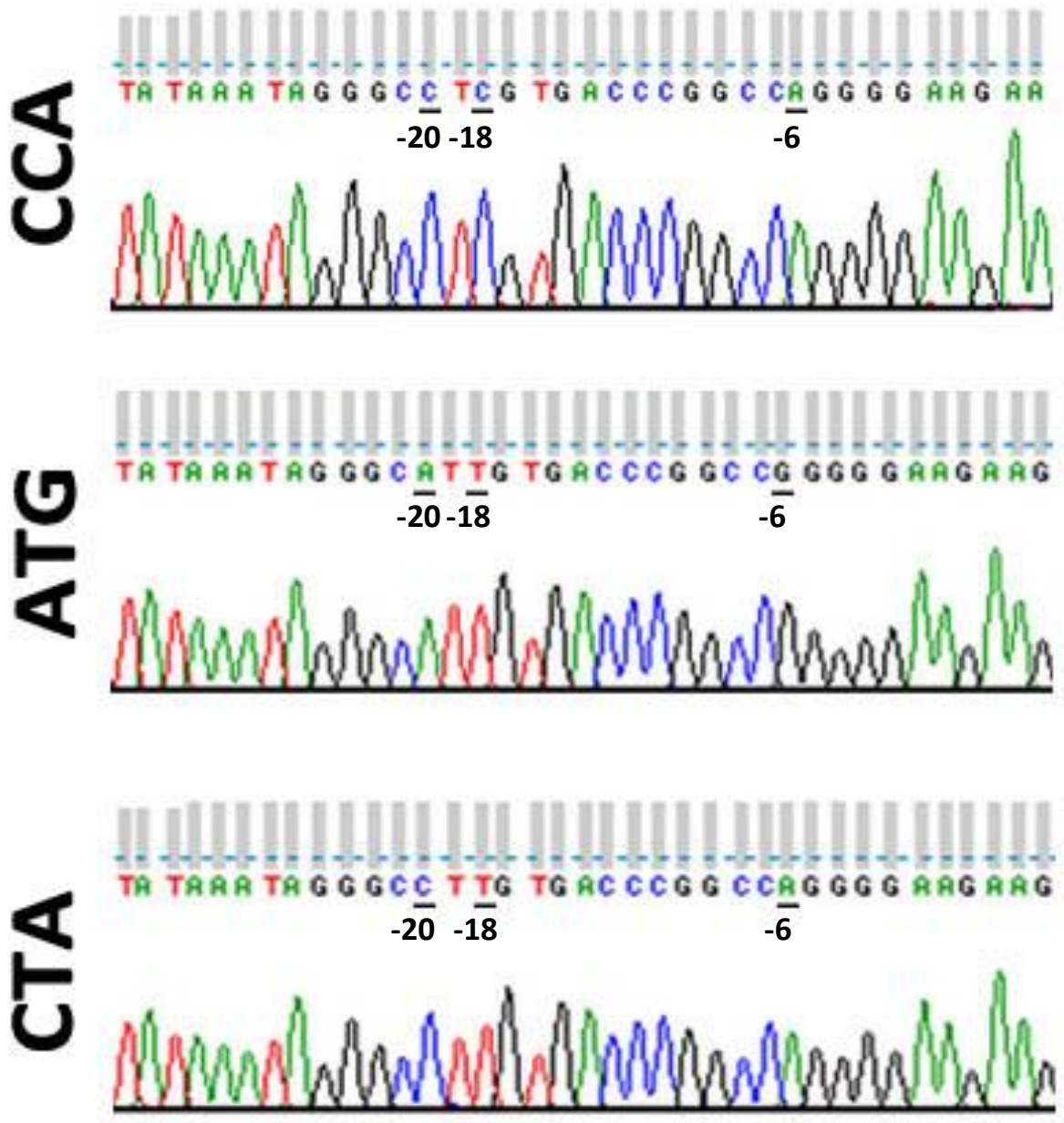

Figure S2. Chromat tracings reveal the results of site-directed mutagenesis in the p0LUC-AGT reporter plasmid containing the SNPs of interest at -20, -18, and -6 from the original plasmid with the CCA haplotype.

REFERENCES

REFERENCES

1. Uhal, B.D., Li, X., Piasecki, C., and Molina-Molina, M. Angiotensin signaling in pulmonary fibrosis. *Int. J. Biochem. Cell Biol.* doi:10.1016/i.biocel.2011.11.019 (2011).

2. Uhal, B.D., Kim, J.K., Li, X., and Molina-Molina, M. Angiotensin-TGF-β1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages. *Curr. Pharm. Des.* 13:1247-1256 (2007).

3. Lindpainter, K., Jin, M., Wilhelm, M.J., Suzuki, F., Linz, W., Schoelkens, B.A., and Ganten, D. Intracardiac generation of angiotensin and its physiologic role. *Circulation.* 77:I18-I23 (1988).

4. Campbell, D.J., Bouhnik, J., Menard, J., and Corvol, P. Identity of angiotensinogen precursors of rat brain and liver. *Nature*. 308:206-208 (1984).

5. Fuxe, K., Bunnemann, B., Aronsson, M., Tinner, B., Cintra, A., von Euler, G., Agnati, L.F., Nakanishi, S., Ohkubo, H., and Ganten, D. Pre- and postsynaptic features of the central angiotensin systems. Indications for a role of angiotensin peptides in volume transmission and for interactions with central monamine neurons. *Clin. Exp. Hypertens. A.* 10:143-168 (1988).

6. Filippatos, G., Tilak, M., Pinillos, H., and Uhal, B.D. Regulation of apoptosis by angiotensin II in the heart and lungs (Review). *Int. J. Mol. Med.* 7:273-280 (2001).

7. Li, X., Rayford, H., Shu, R., Zhuang, J., and Uhal, B.D. Essential role for cathepsin D in bleomycin-induced apotosis of aleveolar eptohelial cells. *Am. J. Physiol.* 287:L46-L51 (2004).

8. Kasper, M., Lackie, P., Hasse, M., Schuh, D., and Müller, M. Immunolocalization of cathepsin D in pneumocytes of normal human lung and in pulmonary fibrosis. *Virchows Arch.* 428:207-215 (1999).

9. Li, X., Rayford, H., and Uhal, B.D. Essential roles for angiotensin receptor AT1a in bleomycin-induced apoptosis and lung fibrosis in mice. *Am. J. Pathol.* 163:2523-2530 (2003).

10. Wang, R., Ramons, C., Joshi, I., Zagariya, A., Pardo A., Selman, M., and Uhal, B.D. Human lung myofibroblast derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides. *Am. J. Physiol.* 277:L1158-L1164 (1999).

11. Li, X., Molina-Molina, M., Abdul-Hafez, A., Ramirez, J., Serrano-Mollar, A., Xaubet, A., and Uhal, B.D. Extravascular sources of lung angiotensin peptide synthesis in idiopathic pulmonary fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* 291:L887-L895.

12. Wang, R., Zagariya, A., Ang, E., Ibarra-Sunga, O.B., and Uhal, B.D. Fas-induced apoptosis of alveolar epithelial cells requires ANGII generation and receptor interaction. *Am. J. Physiol.* 277:L1245-L1250 (1999).

13. Bargout, R., Jankov, A., Dincer, E., Wang, R., Komodromos, T., Ibarra-Sunga, O., Filippatos, G., and Uhal, B.D. Amiodarone induces apoptosis of human and rat alveolar epithelial cells in vitro. *Am. J. Physiol.* 278:L1039-L1044 (2000).

14. Corvol, P., and Jeunemaitre, X. Molecular genetics of human hypertension: role of angiotensinogen. *Endocr. Rev.* 18:662-677 (1997).

15. Li, X., Zhuang, J., Rayford, H., Zhang, H., Shu, R., and Uhal, B.D. Attenuation of belomycin-induced pulmonary fibrosis by intratracheal administration of antisense oligonucleotides against angiotensinogen mRNA. *Curr. Pharm. Design.* 13:1257-1268 (2007).

16. Fukamizu, A., Takahashi, S., Seo, M.S., Tada, M., Tanimoto, K., Uehara, S., and Murakami, K. Structure and expression of the human angiotensinogen gene. *J. Biol. Chem.* 5:7576-7682 (1990).

17. Morgan, L., Pipkin, B., and Kalsheker, N. Angiotensinogen: Molecular Biology, Biochemistry, and Physiology. *Int. J. Biochem. Cell Biol.* 28:1211-1222 (1996).

18. Morris, B.J. Human renin protein and gene structures: present and future targets for renin blockade in treatment of hypertension. *J. Hypertens.* 7:S9-S14 (1989).

19. Jeunemaitre, X. Genetics of the human renin-angiotensin system in *Handbook of Experimental Pharmacology: Angiotensin vol. 163.* Edited by Unger, T., and Schölkens, B.A. 173-206 (Springer, New York, NY, USA 2004).

20. Shimizu, T., Oishi, T., Omori, A., Sugiura, A., Hirota K., Aoyama, H., Saito, T., Sugaya, T., Kon, Y., Engel, J.D., Fukamizu, A., and Tanimoto, K. Identification of cis-regulatory seuqences in the human angiotensinogen gene by transgene coplacement and site-specific recombination. *Mol. Cell. Biol.* 25:2938-2945 (2005).

21. Feldmer, M., Haling, M., Takahashi, S., Mullins, J.J., and Ganten, D. Glucocorticoid- and estrogen-responsive elements in the 5' flanking region of the rat angiotensinogen gene. *J. Hypertens.* 9:1005-1012 (1991).

22. Oishi, T., Date, S., Shimamoto, Y., Saito, T., Hirota, K., Sugaya, T., Kon, Y., Fukamizu, A., and Tanimoto, K. A nuclear receptor, hepatocyte nuclear factor 4, differently contributes to the human and mouse angiotensinogen promoter activities. *J. Recept. Signal Transduct. Res.* 30:484-494 (2010).

23. Shimamoto, Y., Ishida, J., Yamagata, K., Saito, T., Kato, H., Matsuoka, T., Hirota, K., Daitoku, H., Nangaku, M., Yamagata, K., Fujii, H., Takeda, J., and Fukamizu, A. Inhibitory effect of the small heterodimer partner on hepatocyte nuclear factor-4 mediates bile acid-induced repression of the human angiotensinogen gene. *J. Biol. Chem.* 279:7770-7776 (2004).

24. Ray, S., Boldogh, I., and Brasier, A.R. STAT3 NH_2-terminal acetylation is activated by the hepatic acute-phase response and required for IL-6 induction of angiotensinogen. *Gasteroenterol.* 129:1616-1632 (2005).

25. Jain, S., Li, Y., Patil, S., and Kumar, A. HNF-1α plays an important role in IL-6-induced expression of the human angiotensinogen gene. *Am. J. Physiol.* 293:C401-C410 (2007).

26. Jain, S., Shah, M., Li, Y., Vinukonda, G., Sehgal, P.B., and Kumar, A. Upregulation of human angiotensinogen (AGT) gene transcription by interferon-gamma: involvement of the STAT1-binding motif in the AGT promoter. *Biochim. Biophys. Acta.* 1759:340-347 (2006).

27. Uhal, B.D., Zhang, H., Abdul-Hafez, A., Shu, R., and Li, X. Amiodarone induces angiotensinogen gene expression in lung alveolar epithelial cells through activation protein-1. *Basic Clin. Pharmacol. Toxicol.* 100:59-66 (2007).

28. Abdul-Hafez, A., Shu, R., and Uhal, B.D. JunD and HIF-1α mediate transcriptional activation of angiotensinogen by TGF-β1 in human lung fibroblasts. *FASEB J.* 23:1655-1662 (2009).

29. Yanai, K., Saito, T., Hirota, K., Kobayashi, H., Murakami, K., and Fukamizu, A. Molecular variation of the human angiotensinogen core promoter element located between the TATA box and transcription initiation site affects its transcriptional activity. *J. Biol. Chem.* 272:30558-20562 (1997).

30. Inoue, I., Nakajima, T., Williams, C.S., Quackenbush, J., Puryear, R., Powers, M., Cheng, T., Ludwig, E.H., Sharma, A.M., Hata, A., Jeunemaitre, X., and Lalouel, J.M. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. *J. Clin. Invest.* 99:1786-1797 (1997).

31. Schnee, J.M., and Hsueh, W.A. Angiotensin II, adhesion, and cardiac fibrosis. *Cardiovasc. Res.* 46:264-268 (2000).

32. Goto, M., Hoxha, N., Osman, R., Wen, J., Wells, R.G., and Dell, K.M. Renin-angiotensin system activation in congenital hepatic fibrosis in the PCK rat model of autosomal recessive polycystic kidney disease. *J. Pediatr. Gastroenterol. Nutr.* 50:639-644 (2010).

33. Powell, E.E., Edwards-Smith, C.J., Hay, J.L., Clouston, A.D., Crawford, D.H., Shorthouse, C., Purdie, D.M., and Jonsson, J.R. Host genetic factors influence disease progression in chronic hepatitis C. *Hepatology.* 31:828-833 (2000).

34. Marshall, R.P., Gohlke, P., Chambers, R.C., Howell, D.C., Bottoms, S.E., Unger, T., McAnulty, R.J., and Laurent, G.J. Angiotensin II and the fibroproliferative response to acute lung injury. *Am. J. Physiol. Lung Cell Mol. Physiol.* 286:L156-L164 (2004).

35. Molina-Molina, M., Xaubet, A., Li, X., Abdul-Hafez, A., Friderici, K., Jernigan, K., Fu, W., Ding, Q., Pereda, J., Serrano-Mollar, A., Casanova, A., Rodríguez-Becerra, E., Morell, F., Ancochea, J., Picado, C., and Uhal, B.D. Angiotensinogen gene G-6A polymorphism influences idiopathic pulmonary fibrosis disease progression. *Eur. Respir. J.* 32:1004-1008 (2008).

36. Dixon, J.B., Bhathal, P.S., Jonsson, J.R., Dixon, A.F., Powell E.E., and O'Brien, P.E. Pro-fibrotic polymorphisms predictive of advanced liver fibrosis in the severely obese. *J. Hepatol.* 39:967-971 (2003).

37. Chapman, C.M., Palmer, L.J., McQuillan, B.M., Hung, J., Burley, J., Hunt, C., Thompson, P.K., and Beilby, J.P. Polymorphisms in the angiotensinogen gene are associated with carotid intimal-medial thickening in females from a community-based population. *Atherosclerosis.* 159:209-217 (2001).

38. Xiao, F., Wei, H., Song, S., Li, G., and Song, C. Polymorphisms in the promoter region of angiotensinogen gene are associated with liver cirrhosis in patients with chronic hepatitis B. *J. Gastroenterol. Hepatol.* 21:1488-1491 (2006).

39. Uhal, B.D., Wang, R., Laukka, J., Zhuang, J., Soledad-Conrad, V., and Filippatos, G. Inhibition of amiodarone-induced lung fibrosis but not alveolitis by angiotensin system antagonists. *Pharmacol. Toxicol.* 92:81-87 (2003).

40. Li, X., Molina-Molina, M., Abdul-Hafez, A., Uhal, V., Xaubet, A. and Uhal, B.D. Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis. *Am. J. Physiol. Cell Mol. Physiol.* 295:L178-L185 (2008).

41. Abdul-Hafez, A., Shu, R., and Uhal, B.D. JunD and HIF-1alpha mediate transcriptional activation of angiotensinogen by TGF-beta1 in human lung fibroblasts. *FASEB J.* 23:1655-1662 (2009).

42. Wu, K.K. Analysis of protein-DNA binding by streptavidin-agarose pulldown from *Methods in Molecular Biology vol. 338: Gene Mapping, Discovery, and Expression: Methods and Protocols.* Edited by Bina, M. 281-290 (Humana Press Inc., Totowa, New Jersey USA 2006).

43. Dang, M.T., Gu, C., Klavanian, J.I., Jernigan, K.A., Friderici, K.H., Cui, Y., Molina-Molina, M., Ancochea, J., Xaubet, A., and Uhal, B.D. Angiotensinogen promoter polymorphisms predict low diffusing capacity in U.S. and Spanish IPF cohorts. *Lung.* 191:353-360 (2013).

44. Nicholson, A.G., Fulford, L.G., Colby, T.V., du Bois, R.M., Hansell, D.M., and Wells, A.U. The relationship between individual histologic features and disease progression in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care. Med.* 166:173-177 (2002).

45. Kvietikova, I., Wenger, R.H., Marti, H.H., and Gassman, M. The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. *Nucleic Acids Res.* 23:4542-4550 (1995).

46. TRANSFAC BioBase Biological Databases. https://portal.biobase-international.com/cgi-bin/build_t/idb/1.0/searchengine/start.cgi Last visited May 20, 2014.

47. Albá, M.M., Farré, D., García, D., Escudero, R., Nuñez, O., Martínez, J., and Messeguer, X. PROMO. http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3 Last visited May 20, 2014.

48. Dickson, M.E., Tian, X., Liu, X., Davis, D.R., and Sigmund, C.D. Upstream stimulatory factor is required for human angiotensinogen expression and differential regulation by the A-20C polymorphism. *Circ. Res.* 103:940-947 (2008).

49. Qyang, Y., Luo, X., Lu, T., Ismail, P.M., Krylov, D., Vinson, C., and Sawadogo, M. Cell-type-dependent activity of the ubiquitous transcription factor USF in cellular proliferation and transcriptional activation. *Mol. Cell. Biol.* 19:1508-1517 (1999).

50. Samoylenko, A., Roth, U., Jungermann, K., and Kietzmann, T. The upstream stimulatory factor-2a inhibits plasminogen activator inhibitor-1 gene expression by binding to a promoter element adjacent to the hypoxia-inducible factor-1 binding site. *Blood.* 97:2657-2666 (2001).

51. Hellman, L.M., and Fried, M.G. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat. Protoc.* 2:1849-1861 (2007).

CHAPTER 4

PREDICTORS OF POOR PULMONARY FUNCTION IN IPF COHORTS: VARIANTS IN *AGT* AND *TGF-β_1*

Angiotensinogen Promoter Polymorphisms Predict Low Diffusing Capacity in U.S. and Spanish IPF Cohorts*

Abstract.

Background. SNPs in *AGT* at positions -20 and -6 are associated with increased severity and progression of various fibrotic diseases. Our earlier work demonstrated that the progression of IPF was associated with the A-6 allele. This study examined the hypothesis that the homozygous CC genotype at -20 and the AA genotype at -6 would confer worse measures of pulmonary function (as measured by pulmonary function tests) in IPF.

Methods. Multiple logistic regression analysis was applied to a NIH Lung Tissue Research Consortium (LTRC) cohort and a Spanish cohort, while also adjusting for covariates to determine the effects of these SNPs on measures of pulmonary function.

Results. Analysis demonstrated that the CC genotype at -20 was strongly associated with reduced diffusing capacity in males in both cohorts ($p = 0.0028$ for LTRC and $p = 0.017$ for Spanish cohort). In females, the AA genotype was significantly associated with lower FVC ($p = 0.0082$) and V_{alv} ($p = 0.022$). In males, the haplotype CA at -20 and -6 in *AGT* was also strongly associated with reduced diffusing capacity in both cohorts

Conclusions. This study is the first to demonstrate an association of *AGT* polymorphisms (A-20C and G-6A) with lower measures of pulmonary function in IPF. It is also the first to relate the effect of sex in lung fibrosis with polymorphisms in *AGT.*

* From first author paper in *Lung.*

Introduction.

IPF is the most common form of interstitial lung disease. It is a "chronic, progressive, and irreversible" condition with a bias toward males and people in their fifth through eighth decade of life.[1] Upon diagnosis, the mean survival is 3 years. Currently, the only therapy that can prolong survival is lung transplantation, but the 5-year post-operative survival rate is 44%.[1] The other current modes of therapy (corticosteroids and immunosuppressants) are of minimal benefit to IPF patients. This reflects the incomplete knowledge underlying the pathogenesis of IPF and paves the way for novel therapies to address this void.[2]

A strong predictor of mortality in IPF is the number of myofibroblastic foci.[3] Myofibroblasts play an important role in lung fibrosis. They can be derived from a variety of sources, including pericytes, fibrocytes, epithelial or endothelial cells, and normal lung fibroblasts, which, when stimulated with transforming growth factor (TGF)-β_1, differentiate into myofibroblasts. Myofibroblasts within many tissues are a known source of collagen and ANGII, and the ANGII produced by myofibroblasts is known to mediate fibrogenesis in various organ systems such as the heart, kidney, liver, pancreas, skin, and lung.[4-10] ANGII is derived from its precursor AGT, and both AGT and ANGII have been shown by this laboratory to be required for experimental lung fibrosis.[11-12] ANGII also enhances TGF-β_1 synthesis in human lung myofibroblasts isolated from patients with IPF.[9] In turn, TGF-β_1 is able to stimulate *AGT* transcription in myofibroblasts, thus creating an “ANGII-TGF-β_1 autocrine loop” in myofibroblasts.[9] This laboratory also demonstrated that TGF-β_1-inducible *AGT* transcription is

regulated through two transcription factors, JunD and HIF-1α, both of which act on binding domains in the core promoter of *AGT* in the region spanning from −46 to +22 from the transcription start site.[13]

The core promoter also contains three SNPs located at −20, −18, and −6. The SNPs at these locations have been shown to result in changes in *AGT* transcription rate in non-pulmonary cell types. In hepatocytes, the presence of the CC haplotype at −20 and −18 increased *AGT* transcription to more than two-fold when compared to the AT haplotype.[14] Similarly, the presence of the A allele at −6 increased *AGT* transcription in comparison to the G allele at the same position.[15] These SNPs have also been associated with the severity and/or progression of various diseases, including IgA nephropathy, hepatic fibrosis and cirrhosis, hypertension, and IPF.[16-21] In a Spanish IPF cohort, our laboratory demonstrated that the AA genotype of G-6 A was significantly associated with disease progression as measured by alveolar-arterial oxygen gradient over time.[20]

Based on this, it was hypothesized that the presence of the CC genotype at −20 and/or the AA genotype at −6, particularly when found together, would confer worse measures of pulmonary function in IPF as measured by PFTs. In accordance with this hypothesis, it was theorized that the presence of both of these alleles would confer a “risk haplotype” for IPF; the risk haplotype was predicted to be CA (at the −20 and −6 positions, respectively).

Materials and Methods.

Subjects. The LTRC provided 163 samples of purified DNA with over 1,100 associated clinical variables from IPF patients. From these, samples that were unable to be genotyped and samples that were missing variables of interest were excluded. The final pool consisted of 149 samples and 68 variables of interest that came from the categories of demographics, tobacco use, environmental exposure, disease history, medications, pulmonary function tests, and arterial blood gases. This pool was composed of 94 males and 55 females [age = 63.4 ± 8.5 and 62.4 ± 9.2 (mean ± SD), respectively]. Similar analyses were performed on a second cohort consisting of 203 patients from a Spanish population. This group was composed of 123 males and 80 females [age = 66.1 ± 10.6 and 67.5 ± 13.1 (mean ± SD), respectively].

Genotyping Polymorphisms at −20 and −6. The genotyping protocol was derived from Jeunemaitre et al. [22-23] with modifications in primer design. The primers utilized were 5'-GTC GCT TCT GGC ATC TGT CC-3' (forward) and 5'-CCT TTT CCT CCT AGC CCA CA-3' (reverse). Each sample was subjected to the following PCR cycling conditions: 94°C for 5 minutes; followed by 35 cycles of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 45 seconds; with a final extension at 72°C for 7 minutes. Each reaction was performed in a 20-µL volume containing 0.5 U *Taq* polymerase (Promega, Madison, WI), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µg/µL Puregene RNAse A Solution (Gentra Systems, Minneapolis, MN), and 1 µM of each primer. The amplification of each product was checked on a 2% agarose gel using 5 µL of the PCR product. If amplification was sufficient, the remaining 15 µL underwent a purification step to remove

contaminating primers and dNTPs. The purification step consisted of adding 0.45 µL (5 U/µL) of Antarctic Phosphatase (New England Biolabs, Ipswich, MA), 1.5 µL of 10× Antarctic phosphatase buffer (New England Biolabs), and 0.225 µL (10 U/µL) of Exonuclease I (USB Corporation, Cleveland, OH). This mixture was incubated at 37°C for 30 minutes followed by a 20 minute incubation at 80°C. Sequencing was performed using 2 µL of purified PCR product, 9.7 µL of water, and 0.3 µL (100 µM) of primer. Both forward and reverse primers were utilized in separate reactions and sequenced on an ABI Prism 3700 DNA Analyzer (Life Technologies, Carlsbad, CA) at the RTSF at Michigan State University. The results were analyzed using the program Sequencher v4.7 (Gene Codes Corp., Ann Arbor, MI) to determine SNPs rs5050 (A-20C) and rs5051 (G-6A) located in the promoter of *AGT.* Genotyping at -18 revealed a lack of diversity at this position, therefore, it was excluded from this study.

***Statistical Analyses*.** The relationship between measures of pulmonary function (as measured by PFTs, including FEV_1, FVC, FEV_6, mean DL_{CO}, V_{alv}, and K_{CO}) and the genotyped SNPs at −20 and −6 was tested by fitting regression models assuming different gene action modes (i.e., additive, dominance, and recessive) after adjusting for the effects of covariates. Data analysis was done with statistical software R (ver. 2.13.2). From the original 163 individuals, 14 individuals were excluded because their samples were unable to be genotyped or their variables of interest were missing. To account for possible sex differences in disease progression, missing phenotypic values were imputed using the mean value of the corresponding phenotype for male and female data separately. Analysis for the Spanish cohort

was done in a similar manner except for the inclusion of covariates (due to the lack of collection of these variables).

All the phenotypes (as measured by PFTs) were individually analyzed. A stepwise variable selection was initially performed in R on all covariates for each phenotype. The selected covariates were then fitted into the genetic models (see **Supplementary Table S5** in the **Appendix**) together with the SNP variables. For each phenotype, roughly 10-15 covariates were left after variable selection. Three genetic models representing different gene action modes were considered in this study (the joint model analyzing the whole population is in **Supplementary Table S5** in the Appendix). In practice, the true disease model is unknown. Statistically, a model selection criterion can be used to choose which genetic model fits the data best. The Akaike information criterion (AIC) was used to select the optimal model, which is defined as AIC = -2 log L + 2 k, where L is the regression likelihood and k is the total number of parameters fitted in the model. The model with the minimum AIC value was chosen as the optimal one. For the three models, testing a SNP effect is equivalent to testing H_0: $\beta_1 = \beta_{12} = 0$, a 2 degrees-of-freedom (df) likelihood ratio test, while adjusting for the effects of other covariates. The likelihood ratio statistic asymptotically follows a χ^2 distribution with 2 df. For each phenotype, multiple-testing adjustment was done for the two position. Thus, any SNP with $p < 0.025$ was considered statistically significant by maintaining a family-wise error rate of 0.05. To assess whether male and female populations have different genetic bases in determining worse measures in pulmonary functions, the above models were modified by

removing the sex covariate as well as the genetic-by-sex interaction term (the sex-specific model in **Supplementary Table S5**) and were fitted to the male and female data separately. The same set of covariates fitted with the male and female combined data was fitted into the modified models. A likelihood ratio test was applied to test the significance of the regression coefficients after selecting the optimal model using the AIC criterion. A power study revealed that the datasets have > 90% power to detect a mean difference > 0.7 between the largest and smallest means among the three genotype groups with a sample size of 55, the smallest sample size in our study (females in the LTRC cohort).

Results.

***Characteristics of the Patient Population*. Table 4.1** summarizes the age and mean PFT values for the LTRC and the Spanish cohorts for which all genotyping and function test data were available. The data are separated by sex in accordance with the finding of sex-specific differences in the association between *AGT* genotype and PFT values, which is discussed below. No statistically significant differences were observed between males and females in any of the data reported in **Table 4.1**.

***Genotype and Allele Frequencies*.** The genotype and allele frequencies for the A-20C and G-6A polymorphisms in *AGT* are summarized in **Tables 4.2** and **Table 4.3**. No statistically significant differences were observed in the allele frequencies at the −20 or −6 position between men and women (**Table 4.3**).

Table 4.1. Mean values for variables of interest in the LTRC and Spanish cohorts.

	LTRC COHORT		SPANISH COHORT	
CHARACTERISTICS	**Males (*n* = 94)**	**Females (*n* = 55)**	**Males (*n* = 123)**	**Females (*n* = 80)**
Age (years)	63.4 ± 8.5	62.4 ± 9.2	66.1 ± 10.6	67.5 ± 13.1
FEV_1 (L)	2.3 ± 0.6	1.7 ± 0.5	78.6 ± 20.0*	80.2 ± 22.5*
FVC (L)	2.8 ± 0.8	2.0 ± 0.6	70.1 ± 15.7*	71.4 ± 20.1*
FEV_1/FVC (%)	82.7 ± 6.4	83.4 ± 7.2	–	–
FEV_6 (L)	2.8 ± 0.7	2.0 ± 1.0	–	–
PEF (L/s)	8.5 ± 2.2	6.0 ± 1.6	–	–
TLC (% predicted)	–	–	70.5 ± 14.1	69.8 ± 15.7
P_AO (mm Hg)	–	–	71.7 ± 12.8	73.3 ± 14.1
DL_{CO} [mL/(min x mm Hg)]	12.1 ± 4.7	10.0 ± 3.6	56.8 ± 16.2*	56.6 ± 18.3*
V_{alv} (L)	3.9 ± 0.9	3.0 ± 0.6	–	–
K_{CO} [mL/(min x mm Hg x L)]	3.1 ± 0.9	3.3 ± 0.9	78.4 ± 21.0*	77.4 ± 21.3*

Data are presented as mean ± SD; *units are defined as % predicted; 1 mm Hg = 0.133 kPa.

FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity, FEV_6 = forced expiratory volume in 6 seconds, PEF = peak expiratory flow, TLC = total lung capacity, P_AO = alveolar-arterial oxygen tension difference, DL_{CO} = diffusing capacity of the lung for carbon monoxide, V_{alv} = alveolar volume, K_{CO} = ratio between DL_{CO} and alveolar volume.

Table 4.2. Genotype frequencies for *AGT* polymorphisms at A-20C and G-6A in the LTRC and Spanish cohorts.

		% LTRC			% Spanish		
SNP	**Genotype**	**Males (94)**	**Females (55)**	**Total (149)**	**Males (123)**	**Females (80)**	**Total (203)**
A−20C	AA	71.3 (67)	69.1 (38)	70.5 (105)	62.6 (77)	70.0 (56)	65.5 (133)
	AC	26.6 (25)	27.3 (15)	26.9 (40)	33.3 (41)	28.8 (23)	31.5 (64)
	CC	2.1 (2)	3.6 (2)	2.7 (4)	4.1 (5)	1.3 (1)	3.0 (6)
G-6A	GG	28.7 (27)	29.1 (16)	28.9 (43)	28.4 (35)	33.8 (27)	30.5 (62)
	AG	59.6 (56)	50.9 (28)	56.4 (84)	52.3 (65)	47.5 (38)	50.7 (103)
	AA	11.7 (11)	20.0 (11)	14.8 (22)	18.7 (23)	18.8 (15)	18.7 (38)

Data are presented as % (number of individuals from population as categorized by column heading).

Table 4.3. Allele frequencies for *AGT* polymorphisms at A-20C and G-6A in the LTRC and Spanish cohorts.

		% LTRC			% Spanish		
SNP	**Allele**	**Males (*n*)**	**Females (*n*)**	**Total (*n*)**	**Males (*n*)**	**Females (*n*)**	**Total (*n*)**
A−20C	A	84.6 (159)	82.7 (91)	83.9 (250)	79.2 (195)	84.4 (135)	81.3 (330)
	C	15.4 (29)	17.3 (19)	16.1 (48)	20.7 (51)	15.6 (25)	18.7 (76)
G-6A	G	58.5 (110)	54.6 (60)	57.1 (170)	54.9 (135)	57.5 (92)	55.9 (227)
	A	41.5 (78)	45.4 (50)	42.9 (128)	45.1 (111)	42.5 (68)	44.1 (179)

Data are presented as % (number of individuals from population as categorized by column heading).

Influence of AGT Genotypes on PFTs in IPF. In an analysis of each cohort as a whole (**Table 4.4**, i.e., without separation by sex), the CC genotype at −20 was most strongly associated with reduction of K_{CO} in both cohorts. However, the impact of sex on this measure was also significant (see below). The AA genotype at −6 also was associated with reduction of K_{CO}. **Table 4.4** lists only those PFT data for which statistically significant differences were observed in this analysis.

Influence of Sex on AGT Genotypes on PFTs in IPF. When the whole population was reanalyzed with sample separation by sex, several sex-specific effects of *AGT* genotype on PFT values were revealed. In the male IPF population (**Table 4.5**), the CC genotype at the −20 position was associated with a very strong reduction in K_{CO} (from 3.19 ± 0.84 to 1.46 ± 0.34) of high statistical significance ($p = 0.0028$) in the LTRC cohort. This effect was also seen in the Spanish cohort [Table 4.5 ($p = 0.017$)]. The AA genotype at −6 also was associated with reduced K_{CO} in males, but with lower statistical significance ($p = 0.0214$) in the LTRC cohort.

In females (**Table 4.6**), significant associations were seen only at the −6 position; the −20 position had no apparent effect. The AA genotype at −6 was associated with a reduction in FVC ($p = 0.0081$) and V_{alv} ($p = 0.022$) in the LTRC cohort. However, in the Spanish cohort, this genotype was associated with an increase in diffusing capacity ($p = 0.023$). As discussed further below, the lack of decrease in K_{CO} may be related to the large decrease in V_{alv} that is associated with the AA genotype at −6 in females ($p = 0.022$).

Table 4.4. Mean values for PFTs in the whole population at the −20 and −6 positions in the LTRC and Spanish cohorts.

−20 *AGT* SNP in LTRC COHORT					
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**	**SEX EFFECT P-VALUE**
FEV_1 (L)	2.04 ± 0.54	2.11 ± 0.76	1.93 ± 0.96	0.019 (M1)	0.0056
K_{CO} [mL/(min x mm Hg x L)]	3.25 ± 0.83	3.08 ± 0.97	2.62 ± 1.44	0.0094 (M2)	0.0023
−20 *AGT* SNP in SPANISH COHORT					
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**	**SEX EFFECT P-VALUE**
K_{CO} (% predicted)	80.6 ± 20.0	75.8 ± 20.7	74.0 ± 8.9	0.04 (M3)	0.048
−6 *AGT* SNP in LTRC COHORT					
PHENOTYPE	**GG**	**GA**	**AA**	**P-VALUE**	**SEX EFFECT P-VALUE**
–	–	–	–	–	–
−6 *AGT* SNP in SPANISH COHORT					
PHENOTYPE	**GG**	**GA**	**AA**	**P-VALUE**	**SEX EFFECT P-VALUE**
DL_{CO} (% predicted)	58.5 ± 15.2	55.5 ± 15.2	58.8 ± 15.2	0.009 (M2)	0.0031
K_{CO} (% predicted)	79.7 ± 18.4	78.2 ± 20.8	79. ± 21.0	0.027 (M2)	0.0074

Data are presented as mean ± SD. Significant results are accepted with $p < 0.025$ (with Bonferroni correction).

Table 4.5. Mean values for PFTs in the male population at the −20 and −6 positions in the LTRC and Spanish cohorts.

−20 *AGT* SNP IN MALE LTRC COHORT				
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**
FEV_1 (L)	2.21 ± 0.52	2.47 ± 0.65	2.75 ± 0.21	0.0217 (M1)
FEV_6 (L)	2.65 ± 0.55	2.95 ± 0.78	3.45 ± 0.35	0.012 (M1)
FVC (L)	2.71 ± 0.64	2.99 ± 0.56	3.45 ± 0.07	0.019 (M1)
K_{CO} [mL/(min x mm Hg x L)]	3.19 ± 0.84	3.04 ± 0.96	1.46 ± 0.34	0.0028 (M1)
−20 *AGT* SNP IN MALE SPANISH COHORT				
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**
DL_{CO} (% predicted)	57.4 ± 12.3	52.5 ± 16.9	51.6 ± 7.1	0.05 (M3)
K_{CO} (% predicted)	81.3 ± 20.4	72.0 ± 21.2	73.4 ± 9.8	0.017 (M3)
−6 *AGT* SNP IN MALE LTRC COHORT				
PHENOTYPE	**GG**	**GA**	**AA**	**P-VALUE**
K_{CO} [mL/(min x mm Hg x L)]	3.23 ± 0.85	3.06 ± 0.96	3.05 ± 1.1	0.021 (M3)
−6 *AGT* SNP IN MALE SPANISH COHORT				
PHENOTYPE	**GG**	**GA**	**AA**	**P-VALUE**
–	–	–	–	–

Data are presented as mean ± SD. Significant results are accepted with $p < 0.025$ (with Bonferroni correction).

Table 4.6. Mean values for pulmonary function tests in the female population at the −20 and −6 position in the LTRC and Spanish cohorts.

−20 *AGT* SNP IN FEMALE LTRC AND SPANISH COHORTS				
PHENOTYPE	**AA**	**AC**	**CC**	**P-VALUE**
–	–	–	–	–
−6 *AGT* SNP IN FEMALE LTRC COHORT				
PHENOTYPE	**GG**	**GA**	**AA**	**P-VALUE**
FVC (L)	2.08 ± 0.58	2.19 ± 0.50	1.38 ± 0.33	0.0082 (M2)
V_{alv} (L)	3.27 ± 0.54	3.20 ± 0.54	2.50 ± 0.69	0.022 (M2)
−6 *AGT* SNP IN FEMALE SPANISH COHORT				
PHENOTYPE	**GG**	**GA**	**AA**	**P-VALUE**
DL_{CO} (% predicted)	58.1 ± 15.9	56.4 ± 17.3	69.1 ± 13.4	0.011 (M2)
K_{CO} (% predicted)	77.7 ± 18.2	78.4 ± 20.2	90.6 ± 15.0	0.023 (M2)

Data are presented as mean ± SD. Significant results are accepted with $p < 0.025$ (with Bonferroni correction).

Analysis of an "IPF Risk Haplotype." Multiple-position analysis revealed that in males, the *AGT* haplotype CA (at −20 and −6, respectively) was strongly associated with reduced K_{CO} in both the LTRC cohort ($p = 0.0048$) and the Spanish cohort ($p = 0.014$). This association was not statistically significant in females. Interestingly, the AG haplotype at −20 and −6 also was associated with reduced K_{CO} in males in the LTRC cohort, but at a lower statistical significance ($p = 0.031$). When the combined male and female data were analyzed, no significant haplotype was found.

Discussion.

Influence of AGT Genotypes on PFTs in IPF. Given that diffusing capacity for carbon monoxide is the best noninvasive clinical measure of the thickness of the alveolar-capillary diffusion barrier, it was theorized that diffusing capacity would be decreased the most in individuals with *AGT* genotypes already associated with hypertension and/or higher rates of *AGT* transcription in other organs. In males with IPF, this proved to be the case; the lowest K_{CO} values were observed in individuals with the genotypes CC at −20 and AA at −6. The most drastic decrease was observed with the CC genotype at −20, with which the K_{CO} decreased more than two-fold compared to the AA genotype (**Table 4.5**). In males, FEV_1 and FVC also increased, rather than decreased, with the CC genotype at the −20 position; this might be due to more forceful expirations assisted by the increased elastic recoil imparted by the fibrotic lung parenchyma. Unfortunately, it was not possible to explore this hypothesis further with the LTRC

dataset. Regardless, in females with IPF, the lowest FVC and V_{alv} values and highest K_{CO} were observed in individuals with the AA genotype at −6 (Table 6). These data are consistent with our earlier observations.[21]

***Influence of Sex: Effects of AGT SNPs on PFTs*.** IPF is known to affect men more than women, but little is known about the cause of this sex difference. This study is the first to report an association of genetic variants in *AGT* at the −20 and −6 positions, at both the genotype and haplotype level, with sex. On the genotype level, the male sex had a stronger effect at the −20 position, while the female sex imparted a greater effect at the −6 position. Other authors who studied non-pulmonary systems have also observed sex-specific effects of *AGT* variants, e.g., Chapman et al.[24] demonstrated that the −6 position was also more significantly associated with increased carotid intimal medial thickening in the female population. In the present study, haplotype analysis revealed that the IPF "risk haplotype" CA was significant only in males (the AG haplotype was also significant but to a lesser degree). Although other authors have noted an additional, albeit rare, SNP in *AGT* at the −18 position[14], the −18 position was genotyped here but was not analyzed further due to the lack of this variant in the LTRC or Spanish cohort.

***AGT Promoter SNPs and Transcription Rate*.** In studies of *AGT* synthesis by isolated human hepatocytes, SNPs at the −20 and −6 positions influence the transcription rate of AGT mRNA.[14-15] The transcription rate is higher with the C allele at −20 and the A allele at −6. In earlier studies of animal models of lung fibrosis and isolated lung cells, transcription of *AGT* has been shown to be required for the fibrogenic response to bleomycin and for the

apoptotic response of alveolar epithelial cells to a number of profibrotic stimuli.[9,12] Taken together, these findings suggest, and indeed had led us to hypothesize, that higher rates of *AGT* transcription in lung cells imparted by the CA haplotype would lead to worse lung fibrosis in IPF patients, as indicated by reductions in K_{CO}, DL_{CO}, or FVC. As discussed above, most of these effects were found in this study, but in a surprising sex-dependent manner.

Possible Mechanisms Underlying Sex-Specific Effects of AGT Sequence Variants. Hormonal regulatory elements located in the same *AGT* promoter domain as the SNPs studied here also influence the transcription rate of *AGT*. Of particular interest is the estrogen response element that is located in the *AGT* promoter region spanning −11 to −25.[25] Estrogen receptor alpha (ER-α) preferentially binds to the −20 position if the A nucleotide is present and induces an increase in *AGT* transcription by human liver cells.[25] Estrogen also mediates fibrogenesis by up-regulating the transcription of procollagen I and TGF-β_1.[26] TGF-β_1 stimulates fibroblasts to transition into myofibroblasts, which in turn deposit collagen and express *AGT* constitutively.[9,13] Another mechanism that might regulate *AGT* differentially by sex is if the estrogen receptor binding to the *AGT* promoter prevents the binding of other transcription factors that might otherwise up- or down-regulate *AGT* transcription.

Conversely, the binding domain of the orphan receptor Arp-1 shares homology to the binding domain for ER-α.[27]The binding of Arp-1 to this domain reduces estrogen-induced *AGT* transcription.[27] These data suggest that the balance between estrogen and Arp-1 at the −20 position may thus be an influential factor in this sex discrimination. In males, it is

possible that the balance may favor estrogen-induced *AGT* transcription instead of repression by Arp-1. IPF affects people in their fifth to eighth decade of life, and women in these decades tend to be postmenopausal. In this stage estrogen levels drop; this may explain the bias for males at the −20 position. In males with IPF, the K_{CO} decreased with the presence of CC genotype at −20, while in females there was an increase in the K_{CO} at this same position. Thus, the balance between ER-α and Arp-1 may play a role in this difference. This topic will be an interesting issue for future investigation.

Another possible explanation for this sex difference is the potential role of androgens in *AGT* transcription. Throughout the human life span, androgen receptors are expressed in both mesenchymal and epithelial cells. In studies of the prostate gland, ANGII enhanced the expression of androgen receptors through the ANGII type-1 receptor,[28] and one of the downstream effects of this cascade is prostate cell proliferation. If this model is applicable to the lung, modulation of androgen receptors might also contribute to increased severity of IPF in males. For these reasons, the potential role of androgens in the sex differences that *AGT* variants exert on IPF severity will also be an interesting topic for further research.

In this regard it is important to note that if human, mouse, and rat *AGT* promoter sequences are compared, there is relatively low homology between these species in the TGF-β_1 responsive domain of *AGT* between the TATA box and the transcription initiation site.[13] Due to these sequence differences, human lung cells in culture should remain an important model to complement and extend the studies reported here. Moreover, caution should be exercised in

attempts to extrapolate data on the regulation of *AGT* expression obtained from animal models to human lung fibrosis.

Acknowledgments.

This work was supported by the National Institutes of Health Public Health Services Grant PHS HL-45136 (MTD and BU) and by the National Science Foundation Grant DMS-1209112 (CG and YC).

Conflict of Interest.

All authors have no conflicts of interest to disclose.

TGF-β_1 Codon 10 Variant Predicts Low Diffusing Capacity in IPF

Introduction.

IPF is the most common form of interstitial lung disease with an estimated prevalence of 20 per 100,000 in the United States.[1] IPF is a diagnosis of exclusion requiring the histopathological and/or radiologic pattern of usual interstitial pneumonia. Upon diagnosis, the mean survival is 3 years[1] - reflecting the lack of effective therapies to alter the course of the disease. The current prevailing hypothesis underlying the pathogenesis of IPF is that it is a result of abnormal wound healing consisting of persistent injury to AECs, aberrant fibroblast proliferation and the accumulation of extracellular matrix proteins.[29] Our laboratory have demonstrated a role for the ANG system in these three processes due to the profibrotic nature of ANGII. Recent work from our laboratory demonstrated that the CC genotype in *AGT* (the only known precursor to ANGII) at -20 was strongly associated with reduced diffusing capacity in males with IPF from the United States ($p = 0.0028$) and Spain ($p = 0.017$).[30]

Prior work from this laboratory demonstrated that TGF-β_1 increases *AGT* in primary human lung fibroblasts.[9] In the presence of actinomycin D, TGF-β_1 was unable to induce *AGT* transcription, suggesting that TGF-β_1-inducible-*AGT* transcription is transcriptionally regulated.[13] 67% of this induction is mediated by the core promoter of *AGT* spanning from -46 to +22.[13] This region contains binding sites for JunD and HIF-1α. In response to TGF-β_1, there is an increase in binding of HIF-1α and JunD to the *AGT* core promoter. TGF-β_1-inducible-*AGT*

transcription was eliminated with knock-down of both HIF-1α and JunD.[13] Additionally, JunD is required for collagen deposition induced by TGF-β_1.[31]

TGF-β_1 codon 10 (T869C) and codon 25 (G915C) variants in the signal sequence can influence the circulating levels of TGF-β_1.[32] In hepatocytes, the Proline variant at codon 10 (C869) was associated with an increase in the rate of TGF-β_1 secretion.[33] Along similar lines, the Arginine variant at codon 25 (G915) was associated with increase production in TGF-β_1.[34] Xaubet, et. al. demonstrated that the *TGF-β_1* Proline codon 10 variant was associated with disease progression in IPF.[35] Additionally, the combination of the -6 SNP in *AGT* with the *TGF-β_1* codon 25 variant was associated with a higher stage of liver fibrosis than either variant alone.[36] It is hypothesized that the combination of *AGT* and *TGF-β_1* haplotypes will predict lower pulmonary function than either variant alone in IPF.

Materials and Methods.

Subjects. The IPF cohort obtained from the LTRC that was used to study the effects of *AGT* promoter variants on pulmonary function[30] were utilized in this study. From the 149 IPF samples, all but one sample were successful genotyped for the *TGF-β_1* polymorphisms of interest in codon 10 and 25. The final pool was composed of 94 males and 54 females [age in years = 63.4 $\pm$ 8.5 and 62.6 $\pm$ 9.2 (mean $\pm$ SD), respectively)].

Genotyping TGF-β_1 Polymorphisms. 148 IPF patients from the LTRC cohort that were genotyped for SNPs in *AGT* at the -20 and -6 positions[30] were genotyped at codons 10 and 25 in

TGF-β_1. Briefly, PCR amplification was performed using primers flanking codons 10 and 25. The primers are as follows: 5'-TTC AAG ACC ACC CAC CTT CT-3' (forward) and 5'-TCG CGG GTG CTG TTG TAC A-3' (reverse). Each sample was subjected to the following PCR cycling conditions: 94°C for 5 minutes; followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Each reaction was performed in a 20-µL volume containing 0.5 U *Taq* polymerase (Promega, Madison, WI), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µg/µL Puregene RNAse A Solution (Gentra Systems, Minneapolis, MN), and 1 µM of each primer. The amplification of each product was checked on a 2% agarose gel using 5 µL of the PCR product. If amplification was sufficient, the remaining 15 µL underwent a purification step to remove contaminating primers and dNTPs (as previously published).[30] Purified amplification products were sequenced on an ABI Prism 3700 DNA Analyzer (Life Technologies, Carlsbad, CA) at the RTSF at Michigan State University. The results were analyzed using the program Sequencher v4.7 (Gene Codes Corp., Ann Arbor, MI) to determine SNPs present in codon 10 (rs1800470) and codon 25 (rs1800471) in *TGF-β_1*.

Statistical Analyses. Multiple logistic regression analysis was performed to determine the association between results from PFTs and *TGF-β_1* SNPs while also accounting for the effects of covariates (demographics, tobacco use, environmental exposure, disease history, and medications) as published in our recent paper.[30]

Results.

Characteristics of the Patient Population. The mean age and PFT values are summarized in **Table 4.7** for LTRC samples that were able to be genotyped with all available data of interest. The data is separated by sex due to the sex-specific differences that were observed. No statistically significant differences were observed between males and females in any of the reported data in **Table 4.7**.

Table 4.7. Mean values for variables of interest in the LTRC cohort.

CHARACTERISTICS	Males (*n* = 94)	Females (*n* = 54)
Age (years)	63.4 ± 8.5	62.6 ± 9.2
FEV_1 (L)	2.3 ± 0.6	1.7 ± 0.5
FVC (L)	2.8 ± 0.8	2.0 ± 0.6
FEV_1/FVC (%)	82.7 ± 6.4	83.2 ± 7.2
FEV_6 (L)	2.8 ± 0.7	2.0 ± 0.9
PEF (L/s)	8.5 ± 2.2	6.0 ± 1.6
DL_{CO} [mL/(min x mm Hg)]	12.1 ± 4.7	10.0 ± 3.4
V_{alv} (L)	3.9 ± 0.9	3.0 ± 0.6
K_{CO} [mL/(min x mm Hg x L)]	3.1 ± 0.9	3.3 ± 0.9

Data are presented as mean ± SD; *units are defined as % predicted; 1 mm Hg = 0.133 kPa. FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity, FEV_6 = forced expiratory volume in 6 seconds, PEF = peak expiratory flow, TLC = total lung capacity, P_{AO} = alveolar-arterial oxygen tension difference, DL_{CO} = diffusing capacity of the lung for carbon monoxide, V_{alv} = alveolar volume, K_{CO} = ratio between DL_{CO} and alveolar volume (V_{alv}).

Genotype and Allele Frequencies. Genotype and allele frequencies for T869C (codon 10) and G915C (codon 25) variants are summarized in **Table 4.8** and **Table 4.9**. No statistically significant differences were observed in the allele or genotype frequencies at codon 10 and codon 25 between males and females.

Table 4.8. Genotype frequencies for *TGF-β1* polymorphisms at 869 (codon 10) and 915 (codon 25).

SNP	GENOTYPE	MALES (94)	FEMALES (54)	TOTAL (148)
T869C (CODON 10)	TT	31.9 (30)	33.3 (18)	32.4 (48)
	TC	51.1 (48)	46.3 (25)	49.3 (73)
	CC	17.0 (16)	20.4 (11)	18.2 (27)
G915C (CODON 25)	GG	87.2 (82)	87.0 (47)	87.2 (129)
	GC	12.8 (12)	13.0 (7)	12.8 (19)
	CC	0 (0)	0 (0)	0 (0)

Data are presented as % (number of individuals from population as categorized by column heading).

Table 4.9. Allele frequencies for *TGF-β1* polymorphisms at 869 (codon 10) and 915 (codon 25).

SNP	ALLELE	MALES (*n*)	FEMALES (*n*)	TOTAL (*n*)
T869C (CODON 10)	T	57.4 (108)	56.5 (61)	57.1 (169)
	C	42.6 (80)	43.5 (47)	42.9 (127)
G915C (CODON 25)	G	93.6 (176)	93.5 (101)	93.6 (277)
	C	6.4 (12)	6.5 (7)	6.4 (19)

Data are presented as % (number of individuals from population as categorized by column heading).

Influence of TGF-β_1 Genotypes on PFTs in IPF. Analysis of the whole LTRC cohort demonstrated that there was no significant association between the *TGF-β_1* codon 25 variant and measures of pulmonary function (data not shown). However, statistical analysis demonstrated significant association with the *TGF-β_1* codon 10 variant and FEV_1 ($p = 0.0054$), FVC ($p = 0.0052$), V_{alv} ($p = 0.0033$), and K_{CO} ($p = 0.0054$). The impact of sex on FEV_1, FVC, and V_{alv} were also significant [(see below) **Table 4.10**].

Table 4.10. Mean values for pulmonary function tests in the whole population for the *TGF-β_1* codon 10 variant.

PHENOTYPE	TT	CT	CC	P-VALUE	EFFECT P-VALUE*
FEV_1	2.22 ± 0.70	1.98 ± 0.57	2.01 ± 0.51	0.0054	7.78×10^{-16}
FVC	2.70 ± 0.87	2.41 ± 0.76	2.44 ± 0.67	0.0052	1.41×10^{-12}
V_{ALV}	3.83 ± 0.85	3.45 ± 0.94	3.27 ± 0.87	0.0033	2.02×10^{-10}
K_{CO}	3.38 ± 1.00	3.10 ± 0.82	3.07 ± 0.84	0.0054	0.089

* p-value representing effect of sex; data are presented as mean ± SD.

Influence of Sex oon TGF-β_1 Genotypes on PFTs in IPF. The impact of sex was also analyzed in this cohort revealing several male-specific effects of the *TGF-β_1* codon 10 variant on measures of pulmonary function (**Table 4.11**). In males, the CC genotype (Proline/Proline) variant was associated with significant reductions in FEV_1 (from 2.22 ± 0.70 to 2.01 ± 0.51), FVC (from 2.70 ± 0.87 to 2.44 ± 0.67), and V_{alv} (from 3.83 ± 0.85 to 3.27 ± 0.87). Though the influence of sex on K_{CO} missed significance, reanalysis by separating the

cohort by sex demonstrated a significant reduction in K_{CO} with the CC genotype in males (p = 0.0014). There were no significant associations between the *TGF-β_1* codon 10 variant on measures of pulmonary function in the female cohort.

Table 4.11. Mean values for pulmonary function tests in the male population for the *TGF-β_1* codon 10 variant.

PHENOTYPE	TT	CT	CC	P-VALUE
FEV_1	2.56 ± 0.54	2.16 ± 0.56	2.20 ± 0.46	0.0016
FVC	3.14 ± 0.68	2.63 ± 0.77	2.64 ± 0.66	0.004
V_{ALV}	4.28 ± 0.69	3.68 ± 0.97	3.60 ± 0.89	0.0024
K_{CO}	3.43 ± 0.96	2.91 ± 0.79	3.06 ± 0.96	0.0014

Data are presented as mean ± SD.

Discussion.

Influence of Sex: Effects of the TGF-β_1 Codon 10 Variant on PFTs. The sex-specific effects observed at codon 10 in *TGF-β_1* reflects the male bias of IPF - though little is known about the cause of this sex difference. The CC genotype was associated with reduced measures in FEV_1, FVC, V_{alv}, and K_{CO} in the male LTRC cohort. Since IPF is a restrictive type of lung disease, it is predicted that lower measures in FEV_1, FVC, and K_{CO} would be associated with greater disease severity. The lack of significant association with FEV_1/FVC reflects the restrictive nature of this disease, as reductions in both FEV_1 and FVC "normalizes" this ratio . In this fibrotic disease, the accumulation of extra-cellular matrix proteins in the alveoli can complicate the accurate determination of lung volumes.

The codon 10 variant in *TGF-β_1* is associated with progression of IPF as measured by the alveolar-arterial oxygen gradient.[35] In this study, we were unable to assess this parameter due to the lack of values required to calculate the alveolar-arterial oxygen gradient in the LTRC cohort. Similar to our study, Xaubet et al. did not find any associations with the codon 25 variant suggesting that this SNP does not influence the severity or progression of IPF. However, our study is unique in that a sex-effect was observed with the codon 10 variant whereas this was not observed in the study by Xaubet.

Predicted Risk Haplotypes in AGT and TGF-β_1 in IPF. In a recent study, our laboratory discovered that variants in the *AGT* promoter at -20 and -6 were associated with reductions in K_{CO} and that this effect was influenced by sex.[30] From this, we hypothesized that the combination of *AGT* and *TGF-β_1* variants would be associated with worse measures of pulmonary function. However, in this study, we were unable to assess this hypothesis due to the lack of sufficient numbers of patients containing the predicted "risk haplotype" combination (CC/CC in males at *AGT* -20 and *TGF-β_1* codon 10 respectively or AA/CC at *AGT* -6 and *TGF-β_1* codon 10 respectively). In a similar manner, the lack of significant associations with the codon 25 variant may be influenced by the paucity of IPF samples with the predicted risk CC genotype, partly as a result of the low minor allele frequency observed in this population.

TGF-β_1 Variants and Secretion. TGF-β_1 is up-regulated in both human and animal models of IPF.[36-40] This cytokine is implicated in organ fibrosis due to its ability to 1) stimulate extra-cellular matrix deposition, 2) recruit fibroblasts, and 3) induce the transition of fibroblasts

into myofibroblasts.[41] SNPs in the signal sequence of *TGF-β_1* at codon 10 and 25 can influence the secretion of its protein. In hepatocytes, the presence of Proline at codon 10 was associated with increase secretion of TGF-β_1.[42] In breast cancer cells, this variant caused a 2.8-fold increase in secretion.[43] Serum TGF-β_1 concentrations are also significantly elevated in Proline homozygotes compared to Leucine homozygotes at this codon.[44] In the literature, there are also reports that the Leucine codon 10 variant is associated with higher productions of TGF-β_1. These contradictory results may be influenced by cell and tissue specificity and their underlying genetic background. For instance, the presence of the C-509T SNP in the promoter of *TGF-β_1* masks the effect of the Proline codon 10 variant resulting in lower levels of TGF-β_1 secretion.[42] It would be interesting to see if this promoter variant plays a significant role in IPF as a future study.

Possible Mechanisms Underlying Sex-Specific Effects of TGF-β_1 Codon 10 Variant.

Recouvreux et al. describes sex differences in the pituitary TGF-β_1 system with higher active TGF-β_1 levels and activators, MMP-2, $\alpha_V\beta_6$ and $\alpha_V\beta_8$, found in male mice compared to their female counterparts.[45] Additionally, estrogen was a negative regulator in this system suggesting that higher TGF-β_1 concentrations in males are due to low levels of estrogen, thereby increasing the risk of fibrosis in males.[45] Yokota et al. observed that the frequency of the Proline variant in males with myocardial infarction were higher than healthy males - this effect was not observed in the female cohorts.[44] In this same study, *in vivo* serum TGF-β_1 concentrations were also higher in males with myocardial infarction with the Proline/Proline

genotype. The potential role of the TGF-β_1 system as influenced by hormones is an interesting future study that might shed light on the sex-specific differences observed in IPF.

Clinical Implications of *AGT* and *TGF-β_1* Variants in IPF as Biomarkers

Allele Frequencies in the Control and IPF Populations.

Analysis in IPF cohorts from the United States and Spain demonstrated significant associations with the CC genotype at the -20 position, the AA genotype at -6, and the CA haplotype at the -20 and -6 positions in *AGT* respectively with reductions in the diffusing capacity. This relationship was also observed in the United States IPF cohort with the TGF-β_1 codon 10 Proline/Proline (or CC) genotype. When the allele frequencies for these SNPs were analyzed, there were no significant differences observed between the control and IPF populations (**Table 4.12**). Due to the lack of a parallel control cohort from the LTRC, allele frequencies for this population were obtained using the 1000 Genomes Project from a population of Utah residents with Northern and Western European ancestries (CEU) to reflect the ancestry background present in a majority of the LTRC population.[46]

Table 4.12. Alleles frequencies for control and IPF populations from the United States.

-20 SNP in *AGT*		
ALLELE	**CONTROL**	**IPF**
A	0.84	0.84
C	0.16	0.16

-6 SNP in *AGT*		
ALLELE	**CONTROL**	**IPF**
G	0.60	0.57
A	0.40	0.43

***TGF-β_1* CODON 10 SNP**		
ALLELE	**CONTROL**	**IPF**
T	0.61	0.57
C	0.39	0.43

Variants in *AGT* and TGF-β_1 as Genetic Modifiers.

The lack of significant differences in the allele frequencies between the control and IPF populations demonstrated that the presence of these variants do not predict the risk of having IPF. However, it does not exclude the potential of these variants as genetic modifiers in the severity of IPF. For example, cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator gene (*CFTR*). However, differences in the lung disease severity in CF cannot be entirely attributed to the *CFTR* genotype.[47] From twin and sibship studies, it is estimated that the heritability in this variation is at 0.6-0.8 - indicating a strong genetic component.[48] By using family based-haplotype transmission and linear regression analyses, Bremer et al. observed that the CTC haplotype in *TGF-β_1* at -509 (rs1800469), codon 10 (rs1982073), and intron 5 (rs8179181) respectively, was associated with better lung function in CF patients that were non-homozygous for the ΔF508 mutation ($p = 0.0001$).[47] These data support a role of TGF-β_1 variants as genetic modifiers in the lung disease severity in CF and can be extrapolated to study other diseases, such as IPF.

The ANG System as a Pathway in Disease Modification.

The observation that variants in *AGT* and *TGF-β_1* predicted lower diffusing capacity in IPF may reflect the importance of the ANG system as an important pathway in modifying the severity of the disease. The lower diffusing capacity is reflective of the thickened membrane from the fibrotic interstitium due to the accumulation (and/or lack of degradation) of extra-

cellular matrix proteins and the reduction in surface area due to the apoptosis of AECs and delayed repair. The main effector cells implicated in the remodeling process of the interstitium are the myofibroblasts. In addition to inducing the transition of myofibroblasts from fibroblasts, TGF-β_1 can also induce the transcription of *AGT*, the precursor to the profibrotic peptide ANGII. These events represent part of the ANGII-TGF-β_1 cross-talk present within the myofibroblasts and implicate that alterations in the ANG system that favors the production of ANGII will promote the fibrotic response and reflect the severity of IPF. On this basis, variants in *AGT* and *TGF-β_1* that have been shown to affect its rate of production, can be used as functional biomarkers to predict the severity of IPF. Moreover, these biomarkers can help to identify a sub-population of IPF patients who will be responsive to ARBs by helping to limit the effects of ANGII, thereby stabilizing their lung function and disease severity.

APPENDIX

Table S5. Three genetic models used for the association analysis.

MODEL SYMBOL	GENETIC CODING	THE REGRESSION MODEL	
		JOINT MODEL	SEX-SPECIFIC MODEL
M1	S_i = 0, 1, 2 for aa, Aa, AA	$y_i = \mu + \beta_1 S_i + \beta_2 Sex_i + \beta_{12} S_i * Sex_i + \sum_{j=1}^{p} \gamma_j X_{ij} + e_i$	$y_i = \mu + \beta_1 S_i + \sum_{j=1}^{p} \gamma_j X_{ij} + e_i$
M2	S_i = 0, 1, 1 for aa, Aa, AA		
M3	S_i = 0, 0, 1 for aa, Aa, AA		

where y_i $(i = 1, \dots, n)$ is the response measure for individual *i*; μ is the overall mean; β_1 is the genetic effect for SNP S_i and β_2 is the sex effect; β_{12} is the SNP by sex interaction effect; γ_j's are the coefficients for covariate X_{ij} $(j = 1, \dots, p)$; and e_i is the error term. M1, M2 and M3 represent the additive, dominance and recessive model, respectively. Since the response measures the disease severity, we treat allele *A* as the major allele in all coding. Specifically for the SNP at the A-20C position, allele *A* is the major allele, while at the G-6A position, allele *G* is the major allele.

REFERENCES

REFERENCES

1. King, T.E., Pardo, A., and Selman, M. Idiopathic pulmonary fibrosis. *Lancet.* 378:1949-1961 (2011).

2. Datta, A., Scotton, C.J., and Chambers, R.C. Novel therapeutic approaches for pulmonary fibrosis. *Br. J. Pharmacol.* 163:141-172 (2011).

3. Nicholson, A.G., Fulford, L.G., Colby, T.V., du Bois, R.M., Hansell, D.M., and Wells, A.U. The relationship between individual histologic features and disease progression in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care. Med.* 166:173-177 (2002).

4. Weber, K.T., and Sun, Y. Recruitable ACE and tissue repair in the infracted heart. *J. Renin Angiotensin Aldosterone Syst.* 1:295-303 (2000).

5. Yoshiji, H., Kuriyama, S., Yoshii, J., Ikenaka, Y., Noguchi, R., Nakatani, T., Tsujinoue, H., and Fukui, H. Angiotensin-II type I receptor interaction is a major regulator for liver fibrosis development in rats. *Hepatology.* 34:745-7501 (2001).

6. Klahr, S., and Morrissey, J.J. Comparative study of ACE inhibitors and angiotensin II receptor antagonists in interstitial scarring. *Kidney Int. Suppl.* 63:S111-S114 (1997).

7. Ko, S.H., Hong, O.K., Kim, J.W., Ahn, Y.B., Song, K.H., Cha, B.Y., Son, H.Y., Kim, M.J., Jeong, I.K., and Yoon, K.H. High glucose increases extracellular matrix production in pancreatic stellate cells by activating the renin-angiotensin system. *J. Cell. Biochem.* 98:343-355 (2006).

8. Marshall, R.P., Gohlke, P., Chambers, R.C., Howell, D.C., Bottoms, S.E., Unger, T., McAnulty, R.J., and Laurent, G.J. Angiotensin II and the fibroproliferative response to acute lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 286:L156-L164 (2004).

9. Uhal, B.D., Kim, J.K., Li, X., and Molina–Molina, M. Angiotensin-TGF-β1 crosstalk in human idiopathic pulmonary fibrosis: Autocrine mechanisms in myofibroblasts and macrophages. *Curr. Pharm. Des.* 13:1247-1256 (2007).

10. Wang, R., Ibarra-Sunga, O., Verlinski, L., Pick, R., and Uhal, B.D. Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 279:L143-L151 (2000).

11. Li, X., Rayford, H., and Uhal, B.D. Essential roles for angiotensinogen receptor AT1a in belomycin-induced apoptosis and lung fibrosis in mice. *Am. J. Pathol.* 163:2523-2530 (2003).

12. Li, X., Zhuang, J., Rayford, H., Zhang, H., Shu, R., and Uhal, B.D. Attenuation of bleomycin-induced pulmonary fibrosis by intratracheal administration of antisense oligonucleotides against angiotensinogen mRNA. *Curr. Pharm. Des.* 13:1257-1268 (2007).

13. Abdul-Hafez, A., Shu, R., and Uhal, B.D. JunD and HIF-1α mediate transcriptional activation of angiotensinogen by TGF-β1 in human lung fibroblasts. *FASEB* J 23:1655-1662 (2009).

14. Yanai, K., Saito, T., Hirota, K., Kobayashi, H., Murakami, K., and Fukamizu, A. Molecular variation of the human angiotensinogen core promoter element located between the TATA box and transcription initiation site affects its transcriptional activity. *J. Biol. Chem.* 272:30558-30562 (1997).

15. Inoue, I., Nakajima, T., Williams, C.S., Quackenbush, J., Puryear, R., Powers, M., Cheng, T., Ludwig, E.H., Sharma, A.M., Hata, A., Jeunemaitre, X., and Lalouel, J.M. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. *J. Clin. Invest.* 99:1786-1797 (1997).

16. Goto, S., Narita, I., Saito, N., Watanabe, Y., Yamazaki, H., Sakatsume, M., Shimada, H., Nishi, S., Ueno, M., Akazawa, K., Arakawa, M., and Gejyo, F. A(−20)C polymorphism of the angiotensinogen gene and progression of IgA nephropathy. *Kidney Int.* 62:980-985 (2002).

17. Powell, E.E., Edwards-Smith, C.J., Hay, J.L., Clouston, A.D., Crawford, D.H., Shorthouse, C., Purdie, D.M., and Jonsson, J.R. Host genetic factors influence disease progression in chronic hepatitis. *Hepatology.* 31:828-833 (2000).

18. Dixon, J.B., Bhathal, P.S., Jonsson, J.R., Dixon, A.F., Powell, E.E., and O'Brien, P.E. Pro-fibrotic polymorphisms predictive of advanced liver fibrosis in the severely obese. *J. Hepatol.* 39:967-971 (2003).

19. Xiao, F., Wei, H., Song, S., Li, G., and Song, C. Polymorphisms in the promoter region of the angiotensinogen gene are associated with liver cirrhosis in patients with chronic hepatitis B. *J. Gastroenterol. Hepatol.* 21:1488-1491 (2006).

20. Wu, S.J., Chiang ,F.T., Chen, W.J., Liu, P.H., Hsu, K.L., Hwang, J.J., Lai, L.P., Lin, J.L., Tseng, C.D., and Tseng, Y.Z. Three single-nucleotide polymorphisms of the angiotensinogen gene and susceptibility to hypertension: single locus genotype vs. haplotype analysis. *Physiol. Genomics.* 17:79-86 (2004).

21. Molina–Molina, M., Xaubet, A., Li, X., Abdul-Hafez, A., Friderici, K., Jernigan, K., Fu, W., Ding, Q., Pereda, J., Serrano-Mollar, A., Casanova, A., Rodriguez-Becerra, E., Morell, F., Ancochea, J., Picado, C., and Uhal, B.D. Angiotensinogen −6G > A polymorphism influences idiopathic pulmonary fibrosis disease progression. *Eur. Respir. J.* 32:1004-1008 (2008).

22. Jeunemaitre, X. Haplotypes of angiotensinogen in essential hypertension. *Am. J. Hum. Genet.* 60:1448-1460 (1997).

23. Miller, S.A., Dykes, D.D., and Polesky, H.F. A simple salting out procedure of extracting DNA from human nucleated cells. *Nucleic Acids Res.* 16:1215 (1988).

24. Chapman, C.M., Palmer, L.J., McQuillan, B.M., Hung, J., Burley, J., Hunt, C., Thompson, P.L., and Beilby, J.P. Polymorphisms in the angiotensinogen gene are associated with carotid intimal-medial thickening in females from a community-based population. *Atherosclerosis.* 159:209-217 (2001).

25. Zhao, Y.Y., Zhou, J., Narayanan, C.S., Cui, Y., and Kumar, A. Role of C/A polymorphism at −20 on the expression of human angiotensinongen gene. *Hypertension* 33:108-115 (1991).

26. Gharaee-Kermani, M., Hatano, K., Nozaki, Y., and Phan, S.H. Sex-based differences in bleomycin-induced pulmonary fibrosis. *Am. J. Pathol.* 166:1593-1606 (2005).

27. Narayanan, C.S., Cui, Y., Zhao, Y.Y., Zhou, J., and Kumar, A. Orphan receptor Arp-1 binds to the nucleotide sequence located between TATA box and transcriptional initiation site of the human angiotensinogen gene and reduces estrogen induced promoter activity. *Mol. Cell. Endocrinol.* 148:79-86 (1999).

28. Hoshino, K., Ishiguro, H., Teranishi, J., Yoshida, S., Umemura, S., Kubota, Y., and Uemura, H. Regulation of androgen receptor expression through angiotensin II type 1 receptor in prostate cancer cells. *Prostate.* 71:964-975 (2011).

29. Uhal, B.D., Li, X., Piasecki, C., and Molina-Molina, M. Angiotensin signaling in pulmonary fibrosis. *Int. J. Biochem. Cell Biol.* doi:10.1016/i.biocel.2011.11.019 (2011).

30. Dang, M.T., Gu, C., Klavanian, J.I., Jernigan, K.A., Friderici, K.H., Cui, Y., Molina-Molina, M., Ancochea, J., Xaubet, A., and Uhal, B.D. Angiotensinogen promoter polymorphisms predict low diffusing capacity in U.S. and Spanish IPF cohorts. *Lung.* 191:353-360 (2013).

31. Eickelberg, O., Pansky, A., Koehler, E., Bihl, M., Tamm, M., Hildebrand, P., Perruchoud, A.P., Kashgarian, M., and Roth, M. Molecular mechanisms of TGF-(beta) antagonism by interferon (gamma) and cyclosporine A in lung fibroblasts. *FASEB J.* 15:797-806 (2001).

32. Grainger, D.J., Heathcote, K., Chiano, M., Snieder, H., Kemp, P.R., Metcalfe, J.C., Carter, N.D., and Spector, T.D. Genetic control of the circulating concentration of transforming growth factor type beta1. *Hum. Mol. Genet.* 8:93-97 (1999).

33. Gu, X., Ji, X., Shi, L.H., Yi, C.H., Zhao, Y.P., Wang, A.H., Lu, L.G., Yu, W.B., and Gao, C.F. Transforming growth factor beta1 gene variation Leu10Pro affects secretion and function in hepatic cells. *Dig. Dis. Sci.* 57:2901-2909 (2012).

34. Awad, M., El-Gamel, A., Hasleton, P., Turner, D.M., Sinnott, P.J., and Hutchinson, I.V. Genotypic variation in the transforming growth factor-beta1 gene: association with transforming growth factor-beta1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. *Transplantation.* 66:1014-1020.

35. Xaubet, A., Marin-Arguedas, A., Lario, S., Ancochea, J., Morell, F., Ruiz-Manzano, J., Rodriguez-Becerra, E., Rodriguez-Arias, J.M., Inigo, P., Sanz, S., Campistol, J.M., Mullol, J., and Picado, C. Transforming growth factor-β_1 gene polymorphisms are associated with disease progression in idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 168:431-435 (2003).

36. Powell, E.E., Edwards-Smith, C.J., Hay, J.L., Clouston, A.D., Crawford, D.H., Shorthouse, C., Purdie, D.M., and Jonsson, J.R. Host genetic factors influence disease progression in chronic hepatitis C. *Hepatology.* 4:828-833 (2000).

37. Khalil, N., O'Connor, R.N., Unruh, H.W., Warren, P.W., Flanders, K.C., Kemp, A., Bereznay, O.H., and Greenberg, A.H. Increased production and immunohistochemical localization of transforming growth factor-β in idiopathic pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* 5:155-162 (1991).

38. Khalil, N., O'Connor, R.N., Flanders, K.C., and Unruh, H. TGF-β1, but not TGF-β2 or TGF-β3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. *Am. J. Respir. Cell. Mol. Biol.* 14:131-138 (1996).

39. Coker, R.K., Laurent, G.J., Jeffery, P.K., du Bois, R.M., Black, C.M., and McAnulty, R.J. Localisation of transforming growth factor beta1 and beta3 mRNA transcripts in normal and fibrotic human lung. *Thorax.* 56:549-556 (2001).

40. Santana, A., Saxena, B., Noble, N.A., Gold, L.I., and Marshall, B.C. Increased expression of transforming growth factor β isoforms (β_1, β_2, β_3) in bleomycin induced pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* 13:34-44 (1995).

41. Sheppard, D. Transforming growth factor beta: A central modulator of pulmonary and airway inflammation and fibrosis. *Proc. Am. Thorac. Soc.* 3:413-417 (2006).

42. Gu, X., Ji, X., Shi, L.H., Yi, C.H., Zhao, Y.P., Wang, A.H., Lu, L.G., Yu, W.B., and Gao, C.F. Transforming growth factor beta 1 gene variation Leu10Pro affects secretion and function in hepatic cells. *Dig. Dis. Sci.* 57:2901-2909 (2012).

43. Dunning, A.M., Ellis, P.D., McBride, S., Kirschenlohr, H.L., Healey, C.S., Kemp, P.R., Luben, R.N., Chang-Claude, J., Mannerman, A., Kataja, V., Pharoah, P.D., Easton, D.F., Ponder, B.A., and Metcalfe, J.C. A transforming growth factor beta1 signal peptide variant increases secretion in vitro and is associated with increased incidence of invasive breast cancer. *Cancer Res.* 63:2610-2615 (2003).

44. Yokota, M., Ichihara, S., Lin, T.H., Nakashima, N., and Yamada, Y. Association of a T29→C polymorphism of the transforming growth factor-β1 gene with genetic susceptibility to myocardial infarction in Japanese. *Circulation.* 101:2783-2787 (2000).

45. Recourvreux, M.V., Lapyckyj, L., Camilletti, A., Guida, M.C., Ornstein, A., Rifkin, D.B., Becu-Villalobos, D., and Díaz-Torga, G. Sex differences in the pituitary transforming growth factor-β_1 system: studies in a model of resistant prolactinomas. *Endocrinology.* 11:4192-4205 (2013).

46. The 1000 Genomes Project Consortium. An integrated map of genetic variation from 1,092 human genomes. *Nature.* 491:56-65 (2012).

47. Bremer, L.A., Blackman, S.M., Vanscoy, L.L., McDougal, K.E., Bowers, A., Naughton, K.M., Cutler, D.J., and Cutting, G.R. Interaction between a novel TGF-β1 haplotype and CFTR genotype is associated with improved lung function in cystic fibrosis. *Hum. Mol. Genet.* 17:2228-2237 (2008).

48. Vanscoy, L.L., Blackman, S.M., Collaco, J.M., Bowers, A., Lai, T., Naughton, K., Algire, M., McWilliams, R., Beck, S., Hoover-Fong, J., Hamosh, A., Cutler, D., and Cutting, G.R. Heritability of lung disease severity in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 175:1036-1043 (2007).

CHAPTER 5

DOWN-REGULATION OF ACE-2

The Counter-Regulatory Axis in the ANG System.

General Overview.

In IPF, there is an up-regulation in AGT and cathepsin D - both of which are components of the rate-limiting step in the ANG system.[1-3] These two events will favor the production of the profibrotic peptide, ANGII, which was also up-regulated in IPF. Likewise, reductions in the degradation pathway for ANGII can also result in increased levels of this peptide. One enzyme that is responsible for this degradation is ACE-2, which cleaves the octapeptide ANGII to the heptapeptide ANG1-7. Many of the effects mediated by ANG1-7 counter-acts the fibrotic effects of ANGII. In IPF and experimental models of lung fibrosis, this protective enzyme is also down-regulated.[5]

Down-Regulation of ACE-2 In Models of Fibrosis.

In other organ systems, expression of ACE-2 demonstrated protective effects against experimental models of fibrosis. For instance, over-expression of ACE-2 attenuated the development of ANGII-induced myocardial fibrosis in rats as observed with reductions in collagen depositions. Additionally, reductions in cardiac hypertrophy were also observed in these rats. In the unilateral ureteral obstruction model for nephropathy in mice, the loss of ACE-2 enhanced tubulointerstitial fibrosis.[6] In the bleomycin model of pulmonary fibrosis in mice, there were significant reductions in ACE-2 at the mRNA, protein, and enzymatic activity levels.[5] The additional loss of ACE-2 with a synthetic competitive inhibitor, DX-600 or siRNAs

against ACE-2 enhanced collagen accumulation and ANGII levels within the lung.[5] Administration of recombinant ACE-2 reduced the bleomycin-induced collagen accumulation in these mice.[5] These data suggest that ACE-2 can function to limit the accumulation of ANGII thereby limiting the fibrotic response.

In addition to limiting the local accumulation of ANGII, ACE-2 can also exert its anti-fibrotic effects through its product, ANG1-7. One of the critical events in the abnormal wound healing response underlying the pathogenesis of IPF is the apoptosis of AECs. In cell culture experiments with human AECs, the inhibition of ACE-2 and bleomycin treatment enhanced apoptosis as measured by nuclear fragmentation and casapse-9 levels.[7] However, these apoptosis markers were significantly reduced in the presence of ANG1-7.[7] Current work in our laboratory are ongoing in investigating the signaling mechanisms by which ANG1-7 inhibits apoptosis.

Potential Therapeutic Options.

ACE-2 and its product ANG1-7 are key counter-regulatory components against the fibrotic effects of ANGII. Therefore, understanding the mechanisms by which this counter-regulatory axis is down-regulated will provide further insight into the roles of the ANG system in pulmonary fibrosis. Moreover, it will provide additional therapeutic strategies that are aimed at increasing levels of ACE-2 and ANG1-7 in IPF patients as a means to limit the accumulation of ANGII. Human recombinant ACE-2 is in current development by Apeiron Biologics and GSK for

acute lung injury patients. Additionally, an ANG1-7 receptor agonist (mas agonist) has been patented for treating acute lung injury. The rest of this chapter will focus on two possible ways that ACE-2 is down-regulated in AECs.

Manipulation of the ANG System Abrogates G100S SP-C-Induced Apoptosis of Alveolar Epithelial Cells

Introduction.

The current underlying hypothesis for the pathogenesis of pulmonary fibrosis is that it is a result from abnormal wound healing. Repetitive injury to the alveolar epithelium and the resulting apoptosis of AECs are critical events in this disease process. Earlier work from this laboratory demonstrated that in response to apoptotic inducers, bleomycin, Fas ligand, or TNF-α, AECs synthesize the profibrotic peptide, ANGII from the precursor AGT.[8-11] Additionally, oligonucleotides against *AGT*, neutralizing antibodies against ANGII, or antagonists against the ARs inhibited apoptosis mediated by these inducers.[8, 11] The profibrotic ANGII axis can be counter-regulated by the ANG1-7/ACE-2/mas axis. ACE-2 converts ANGII to the anti-fibrotic peptide, ANG1-7 and ANG1-7 mediates its protective effect through the mas receptor. In human and experimental lung fibrosis, ACE-2 is down-regulated but protective.[4]

ER-stress can also result in the apoptosis of AECs. Mutations in the BRICHOS domain of surfactant protein C (SP-C) can induce ER-stress and subsequently lead to pulmonary fibrosis. At least 10 pathogenic mutations in this domain are related to diffuse interstitial lung diseases and all of which results in AEC death.[12] SP-C is uniquely expressed in type II AECs and mutations in the BRICHOS domain result in misfolding of the protein and activation of the UPR.

From this, we hypothesize that ER-stress-induced apoptosis of AECs mediated by BRICHOS domain mutations in SP-C may be regulated by the ANG system and that manipulation of this system can prevent the apoptosis of AECs.

Materials and Methods.

Cell Culture. Human type II AECs cell line A549 were obtained from ATCC (Manassas, VA) and cultured in F12 medium supplemented with 10% FBS and 1% penicilllin/streptomycin (complete F12 media). Experiments utilized cells that were cultured on 6- or 24-well plates. Before treatment, cells underwent 3 washes with serum-free medium followed by a 24-hour serum starvation. In studies utilizing multiple treatment, cells were exposed to A779 (Sigma-Aldrich, St. Louis, MO) or TAPI-2 (at a final concentration of 2 μM, Calbiochem, Billerica, MA) 30 minutes before transfection. After 4 hours, the transfection solution was replaced with serum-free media. In experiments utilizing TAPI-2, cells were exposed to a second treatment during this period. Freshly prepared A779 or ANG1-7 was replaced every three hours until harvesting to compensate for the low biological half-life of these peptides.

G100S Mutant and Wild-Type SP-C Plasmids. The DNA sequences for the human wild-type (WT) and G100S mutant SP-C carried in the pIRES-dsRED plasmid were constructed in the Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki Japan.[13] The G100S- and WT-containing plasmids were amplified using the Plasmid Plus Maxi Kit (Qiagen, Valencia CA). The manufacturer's protocol was modified to obtain the highest yield

of plasmid DNA possible. The WT and mutant SP-C sequences were verified by sequencing at the Genomics Core at the RTSF at Michigan State University by using the following primers: forward 5′-GACTTTCCAAAATGTCGTAACAACT-3′ and reverse 5′-AAGCGGCTTCGGCCAGTAACGTTA-3' (see **Supplementary Figure S2**).[13]

Transfection of SP-C Plasmids. A549 cells were seeded into 6- or 24-well plates to a density of 75% confluence in complete F12 medium. After 24 hours, the cells were serum starved for 24 hours before transfection. The cells were transfected at a ratio of 0.50 μg plasmid DNA to 1.875 μL Lipofectamine 2000 (Invitrogen Life Technologies, Grand Island, NY). 50 μL of the transfection solution was added to each well in a drop-wise manner. The cells were incubated at 37°C with 5% CO_2; after 4 hours, the medium with the transfection solution was removed and replaced with 0.5 mL or 2 mL of serum-free medium (for 24- and 6-well plates respectively). At this time, 5 μL of a stock solution of saralasin or ANG1–7 and/or A779 was added to the desired wells for a final concentration of 50 μg/mL and 1×10^{-7} M, respectively. Cells were placed back in the incubator. Every 3 hours, ANG1–7 and A779 were replaced at the same final concentration as mentioned above. At 28 hours, the plates were removed from the incubator and assayed for immunoreactive protein or nuclear fragmentation.

Detection of Nuclear Fragmentation. Usually, the detection of nuclear fragmentation involves the use of propidium iodide. However, the fluorescence from the SP-C reporter plasmid was used as an alternative to detect fragmented nuclei. In these assays, detached cells were retained by centrifugation of the 24-well plates during fixation with 70% ethanol. Cells

with discrete nuclear fragments containing condensed chromatin were scored as apoptotic. Apoptotic cells were scored over a minimum of four separate microscopic fields from each of at least three wells per treatment group. As in earlier publications, equating fragmented nuclei with apoptosis was verified by in situ end labeling of fragmented DNA.[14]

Western Blotting. Cells were harvested in an NP-40 based lysis buffer containing protease inhibitors. A BCA assay was performed to determine the protein concentration of each sample. 40 μg of proteins were denatured and ran on Tris-HCl polyacrylamide gels at 120 V. This was followed by a transfer to PVDF membranes for 90 minutes at 100 V. Membranes were washed 3x in TBS containing 0.1% Tween (TBST) before blocking in 5% non-fat dry milk for 60 minutes at room temperature. Membranes were incubated with primary antibodies at 4°C overnight. These antibodies were against SP-C (Santa Cruz Technology, Santa Cruz, CA), BiP (Cell Signaling Technology, Beverly, MA), ACE-2 (Abcam, Eugene, OR), and β-actin (Cell Signaling Technology, Beverly, MA). After overnight incubation, membranes were washed 4x in TBST buffer before incubation with their respective secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized by ECL detection systems (ThermoScientific, Rockford, IL). Densitometry was used to quantitate the bands using ImageJ (NIH).

Results.

G100S SP-C Mutation Induces ER Stress. AECs containing the G100S mutation had significant increases in one of the ER-stress markers, BiP/GRP-78 (**Figure 5.1**). This finding is in agreement with other BRICHOS domain SP-C mutations such as △exon 4 (a splice deletion in exon 4 resulting from an A to G substitution in the first base of intron 4) and L188Q.[15-17]

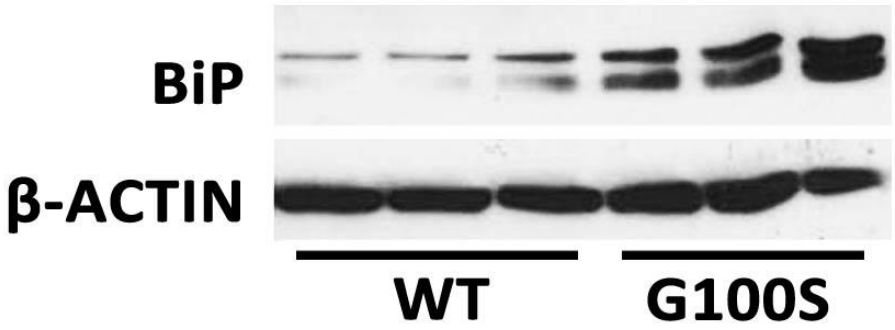

Figure 5.1. G100S SP-C mutation increases BiP/GRP-78, a marker for ER-stress.

G100S SP-C Mutation Affects ACE-2. AECs transfected with the G100S mutation had significant reductions in the anti-fibrotic protein, ACE-2 compared to cells containing the WT SP-C (**Figure 5.2A**). It was hypothesized that this down-regulation was due to ACE-2 ectodomain shedding mediated by ADAM17/TACE (TNF-α converting enzyme). Treatment of AECs with TAPI-2 (an inhibitor of ADAM17/TACE) abrogated the G100S-induced loss of ACE-2 (**Figure 5.2B**).

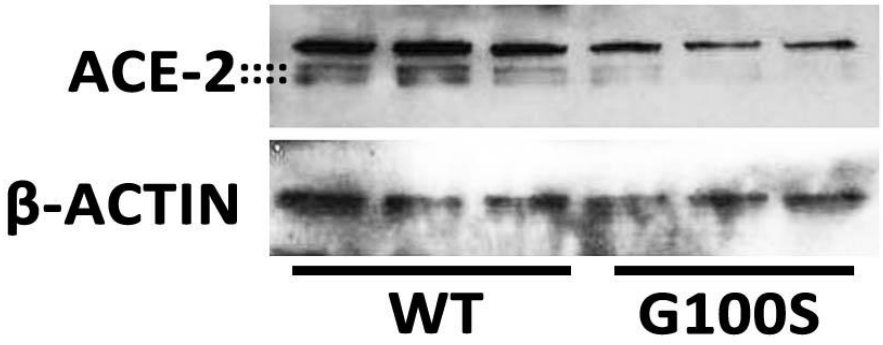

Figure 5.2A. G100S SP-C mutation decreases cellular ACE-2.

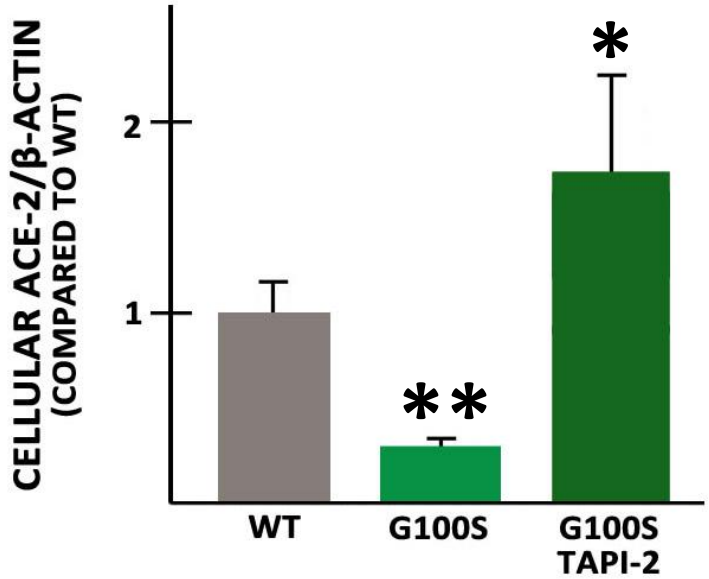

Figure 5.2B. TAPI-2, an inhibitor of ADAM17/TACE abrogates G100S-induced loss of cellular ACE-2. Bars are means ± SEM of ≥ 3 cell cultures. ∗∗ $p < 0.01$ WT vs. G100S, ∗ $p < 0.05$ G100S vs. G100S + TAPI-2 using Student-Newman-Keuls post-hoc test.

G100S SP-C Mutation Induces Apoptosis. Transfection of either the WT or G100S mutation plasmids into AECs showed similar expression of SP-C (**Figure 5.3**). AECs containing the G100S mutation had a significant increase in the number of apoptotic cells as measured by fragmented nuclei (**Figure 5.4**). However, this increase was eliminated with either Saralasin (a

non-selective ARB) or synthetic ANG1-7 (a protective cleaved product of ANGII). ANG1-7 was unable to abrogate apoptosis in the presence of its receptor antagonist, A779.

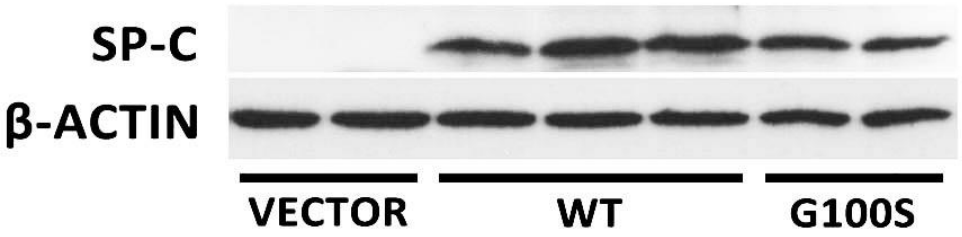

Figure 5.3. Transfection of WT or G100S SP-C plasmids in AECs results in equal expression of the protein.

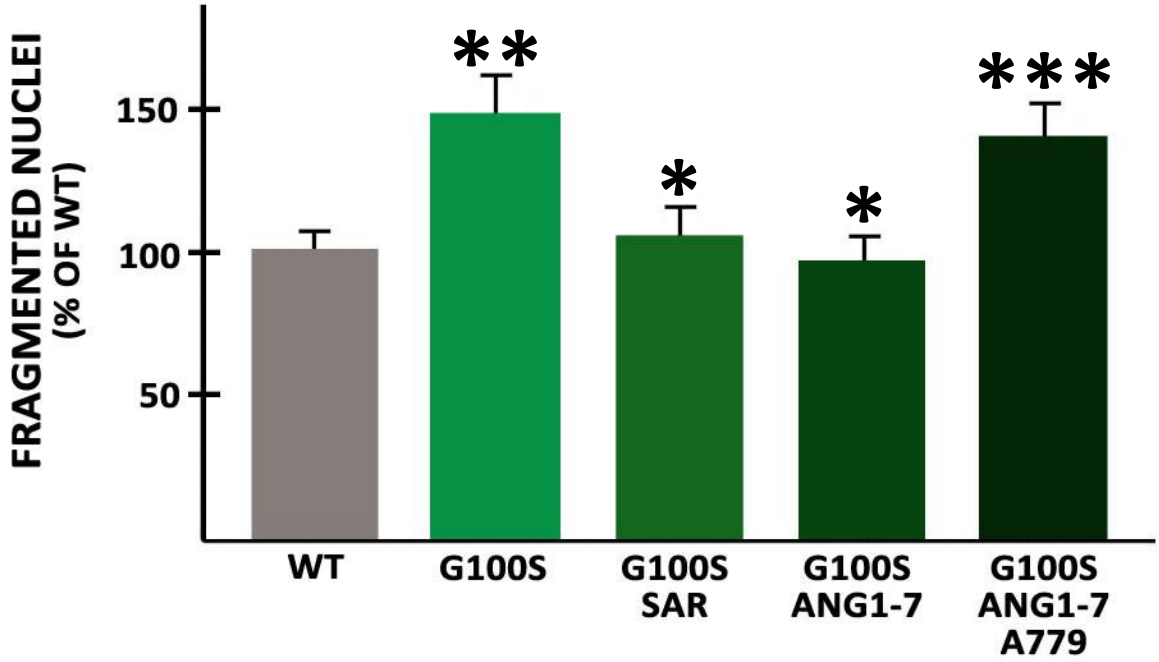

Figure 5.4. Manipulation of the ANG system alters G100S-induced AEC apoptosis. The abrogation of G100S-induced AEC apoptosis as measured by fragmented nuclei by saralasin and ANG1-7 suggests that this event is mediated by the angiotensin and mas receptors. ** $p < 0.01$ WT vs. G100S; * $p < 0.05$ G100S vs. G100S + SAR or G100S + ANG1-7; *** $p < 0.05$ G100S + ANG1-7 vs. G100S + ANG1-7 + A779 using Student-Newman-Keuls post-hoc test. Bars are means ± SEM; SAR = saralasin; ANG1-7 = angiotensin 1-7, A779 = mas receptor antagonist.

Discussion.

The BRICHOS domain in SP-C functions with chaperone-like properties to ensure proper folding and final insertion of the mature protein into the membrane.[18] Mutations in this 100 amino acid region are associated with familial ILD.[13, 19-20] SP-C is specifically produced by type II AECs and these mutations result in their apoptosis secondary to ER-stress and the UPR supporting the Witschi Hypothesis.[12, 17] The Witschi Hypothesis states that the inability of AECs to repair the alveolar epithelium after injury is sufficient to induce fibrosis. The use of caspase inhibitors and deletion of genes involved in apoptosis inhibited experimental lung fibrosis, further supporting this hypothesis.[21-23]

The G100S SP-C mutation was first discovered by Ono et al. in a Japanese kindred with familial pulmonary fibrosis.[13] This mutation resulted in higher levels of ER-stress markers, BiP, IRE-1α, and phospho-PERK compared to the WT SP-C.[13] The latter two proteins are 2 of 3 proximal sensors for the UPR. In our current study, we also observed an increase in BiP, a chaperone protein involved in ER-stress. Additionally, AECs containing this mutation had about a 1.5-fold higher rate of apoptosis ($p < 0.01$) and 3-fold reduction in ACE-2 protein ($p < 0.01$). ACE-2 is an anti-fibrotic enzyme due to its ability to cleave the profibrotic peptide, ANGII into ANG1-7.

Previous work by our laboratory demonstrated that the induction of AEC apoptosis by bleomycin, Fas ligand, or TNF-α was initiated by an increase in *AGT* transcription, the precursor to ANGII.[8-11] These cells also contain the necessary enzymes to convert AGT into ANGII and the

angiotensin receptors to mediate the signaling.[14] This ANGII-producing axis can be counter-regulated by the ANGII-degrading axis, ACE-2/ANG1-7/mas. ACE-2 cleaves ANGII into ANG1-7 and ANG1-7 mediates its anti-fibrotic effect through the receptor mas. The abrogation of the apoptosis of AECs induced by the G100S mutation by ANG1-7 or saralasin, a non-selective ARB, demonstrates a beneficial role in manipulation of this system. It also provides support that ANG1-7 mediates its effect through the mas receptor due to the inability of ANG1-7 to inhibit apoptosis in the presence of A779, a mas receptor antagonist.

The down-regulation of ACE-2 mediated by the G100S mutation was abolished by an ADAM17/TACE inhibitor, TAPI-2. This supports a role of ACE-2 ectodomain shedding as a means to its down-regulation. In the heart, ANGII promotes the activity of ADAM17/TACE resulting in the shedding of ACE-2 with a decrease in cellular ACE-2 and a complementary increase in the extra-cellular space.[24] In patients with heart failure, higher levels of plasma ACE-2 are associated with worsening clinical status.[25] In this study, the extra-cellular levels of ACE-2 was not measured but would be an interesting topic to explore in the future.

In summary, this study revealed that the apoptosis of AECs secondary to the ER-stress induced by the G100S SP-C mutation involved the ANG system. Additionally, apoptosis was blocked by a non-selective ARB and ANG1-7, the cleaved product of ANGII by ACE-2. These findings suggest that manipulation of the ANG system with ARBs, ACE-2, or ANG1-7 can hold therapeutic potential for pulmonary fibrosis - currently, a disease without effective treatment.

Cell-Cycle Dependence of ACE-2 In Alveolar Epithelial Cells*

Introduction.

In the fibrotic lung, the alveolar epithelium is often described as being a "hyperplastic epithelium" due to the excess proliferation of type II AECs from ongoing injury. In this scenario, the epithelium is also referred to as being cuboidal due to the morphology of the type II AECs. In the normal lung, the majority of the surface area of the alveolar epithelium are lined by squamous type I AECs, the differentiated state of type II AECs. In this case, the epithelium can be described as quiescent due to the lack of proliferation in the absence of injury. Unresolved injury to alveolar epithelium is a critical event underlying abnormal wound healing - the prevailing hypothesis for the pathogenesis of pulmonary fibrosis. One of the pathways believed to be involved in abnormal wound healing is the ANG system.

In response to a variety of apoptotic inducers, the apoptosis of AECs require AECs to synthesize AGT and its active peptide, ANGII.[8-11] However, the fibrotic effects of ANGII can be counter-regulated by the ACE-2/ANG1-7/mas axis, where ACE-2 converts ANGII into ANG1-7 and the anti-fibrotic effects of ANG1-7 are signaled through the mas receptor. However, in human and experimental lung fibrosis, this protective enzyme is down-regulated.[4] In AECs, ACE-2 and its product ANG1-7 are protective against apoptosis.[5]

* Adapted from the co-authored paper entitled, Cell Cycle Dependence of ACE-2 Explains Downregulation in Idiopathic Pulmonary Fibrosis, *Eur. Respir. J.* 42:198-210 (2013). Data presented are from a collaborative effort by Dang, M.T., Dang, V., Markey, J., and Piasecki, C.C.

From this, we hypothesized that in pulmonary fibrosis, the down-regulation of ACE-2 is influenced by the cell cycle progression of type II AECs.

Materials and Methods.

Cell Culture. The human lung adenocarcinoma cell line, A549 (ATCC, Manassas, VA) was cultured in F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin (complete F12 media). Cells remained in complete F12 media throughout the treatment period with fresh complete media every 24 hours. Treatment with inhibitors of JNK (SP-600125, Sigma-Aldrich, St. Louis, MO), ERKs (PD-98059, Invitrogen, Grand Island, NY), or p38 (SB-203580, Cell Signaling Technology, Danvers, MA) at a final concentration of 10 μM were applied on cells one day after they reached 100% confluency. Cells were harvested at the 5-day post-confluent state.

RNA Isolation and RT-PCR. A549s were seeded into 6-well plates and harvested at sub-confluent (60-75%) and post-confluent densities in 1 mL of Trizol Reagent (Invitrogen) according to the manufacturer's protocol. From 1 μg of total RNA, first strand cDNA was synthesized using the following reagents: dNTPs, Superscript II Reverse Transcriptase, oligo dT_{12-18}, 5x First Strand Buffer, DTT, and RNaseOUT. 50 ng of total RNA was used for real-time RT-PCR with the SYBR Green PCR Kit (Applied Biosystems, Foster City, CA) and 0.2 μM of primers for human *ACE-2*: - forward: 5'-CAT TGG AGC AAG TGT TGG ATC TT-3' and reverse: 5'-GAG CTA ATG CAT GCC ATT CTC A-3' and human *β-actin* - forward: 5'-AGG CCA ACC GCG AGA AGA TGA CC-3' and reverse: 5'-GAA GTC CAG GGC GAC GTA GC-3'. Each sample was subjected to the following PCR

thermal profile: 95°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds terminating with the dissociation curve analysis (95°C for 1 minute, 55°C for 30 seconds, and 95°C for 30 seconds). The comparative CT method (fold-change = $2^{-\triangle\triangle CT}$; $\triangle CT = CT_{AGT} - CT_{\beta\text{-}ACTIN}$; $\triangle\triangle CT = \triangle CT_{TREATMENT} - \triangle CT_{CONTROL}$) was used to obtain the relative fold change in *ACE-2* expression (normalized to *β-actin*).

Western Blotting. Cells were harvested from 6-well plates in 200 μL of ice-cold NP-40 lysis buffer supplemented with EDTA-free protease inhibitors (Roche, Indianapolis, IN). The protein concentration of each sample was determined using the BCA Assay (ThermoScientific, Rockford, IL). 20 μg of cell lysates were loaded into 10% Tris-HCl polyacrylymide gels in 1x Tris-Glycine-SDS Buffer (Bio-Rad, Hercules, CA, USA) and ran at 120 V. This was followed by transfer of the gel onto PVDF membrane for 90 minutes at 100 V. The membrane was blocked with 5% non-fat dry milk in TBST for 1 hour before incubation with primary antibody overnight at 4°C. The following antibody dilutions were used: ACE-2 at 1:2,000 (Abcam), β-actin at 1:2,000 (Cell Signaling Technologies), and anti-rabbit-HRP at 1:10,000 (Santa Cruz Biotechnology). Bands were chemiluminescently visualized using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoScientific, Rockford, IL). Densitometry using ImageJ (NIH) was used to quantitate the bands.

ACE-2 Enzymatic Activity. Cells were harvested from 6 well plates in ice-cold Tris-HCl buffer (pH = 6.5) containing EDTA-free protease inhibitors (Roche, Indianapolis, IN) and Lisinopril (at a final concentration of 50 μg/L; Sigma-Aldrich, St. Louis, MO) to block ACE activity. After harvesting, samples were immediately prepared for the enzymatic assay. In a half-area black 96-well microtiter plate (Corning, Tweksbury, MA) sitting on ice, the fluorogenic peptide substrate for ACE-2, MCA-YVADAPK (at a final concentration of 10 μM, R&D Systems, Minneapolis, MN) was added to 30 μL of freshly harvested cell lysate in a total volume of 50 μL. Right before the fluorescent reading, the synthetic competitive inhibitor of ACE-2, DX-600 was added to half of the samples to compare the ACE-2 inhibitable activity. The plate was warmed to room temperature and the fluorescence was read for 30 minutes (310/20 nm excitation and 420/50 nm emission). Kinetic readings were normalized to protein concentration (determined using the BCA Assay).

Results.

Cell Cycle State and ACE-2. Manipulation of cell cycle status was performed with different plating densities of A549s. Sub-confluent cells had a higher percentage of bromodeoxyuridine (BrdU) positive nuclei compared to their post-confluent counterparts, indicating higher proliferation rates at sub-confluent densities.[26] These proliferating cells also had less ACE-2 protein (**Figure 5.5**), enzymatic activity ($p < 0.01$, **Figure 5.6**) and mRNA ($p =$

0.0087, **Figure 5.7**) compared to quiescent post-confluent cells. Additionally, the down-regulation of ACE-2 from a quiescent to proliferative state was transcriptionally regulated.[26]

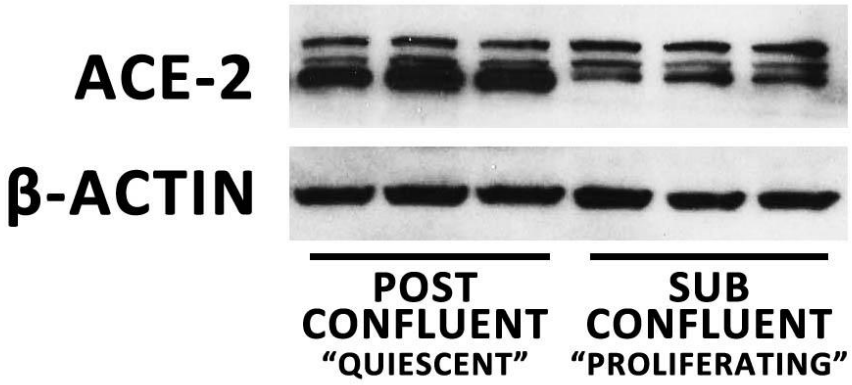

Figure 5.5. Proliferating AECs produce less ACE-2 than quiescent cells.

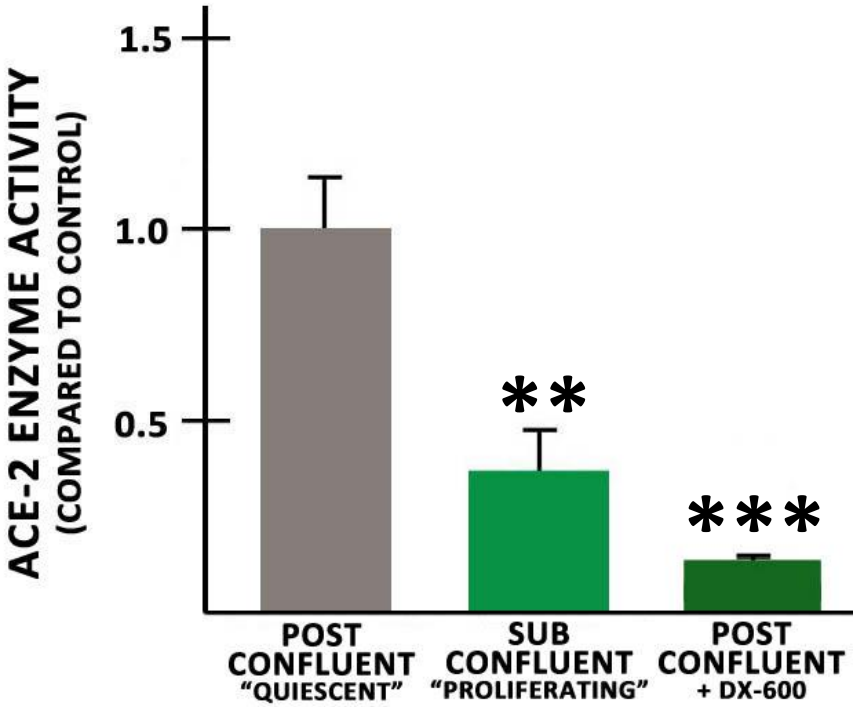

Figure 5.6. Quiescent AECs have more ACE-2 enzymatic activity than their proliferating counterparts (∗∗ p < 0.01). More than 85% of this activity was inhibited with DX-600, a synthetic competitive inhibitor of ACE-2 (∗∗∗ p < 0.01).
Student-Newman-Keuls Analysis with n =3.

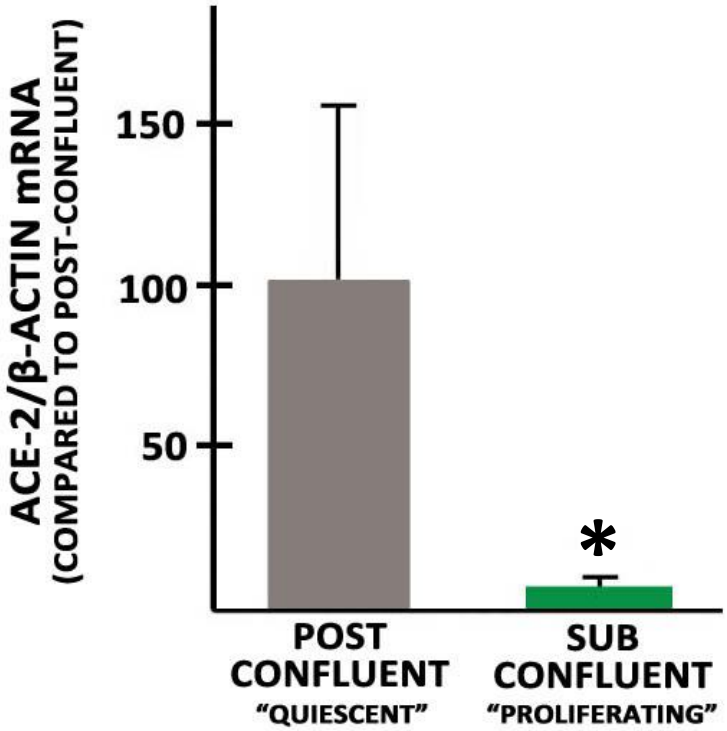

Figure 5.7. ACE-2 mRNA was also elevated in post-confluent quiescent cells (∗ p = 0.0087). Bars represent mean ± SEM with n = 6 using Mann-Whitney Analysis.

JNK Mediated Control of ACE-2. Post-confluent A549s treated with SP-600125, a JNK inhibitor, prevented the accumulation of ACE-2 (data from Piasecki, C.C. in **Figure 5.8A**), whereas inhibitors against extracellular signal-regulated kinases (ERKs) or p38 had no effect on ACE-2 protein levels in quiescent cells (**Figure 5.8B** and **Figure 5.8C**). This suggests that the up-regulation in ACE-2 during the quiescent state is mediated by JNK in AECs.

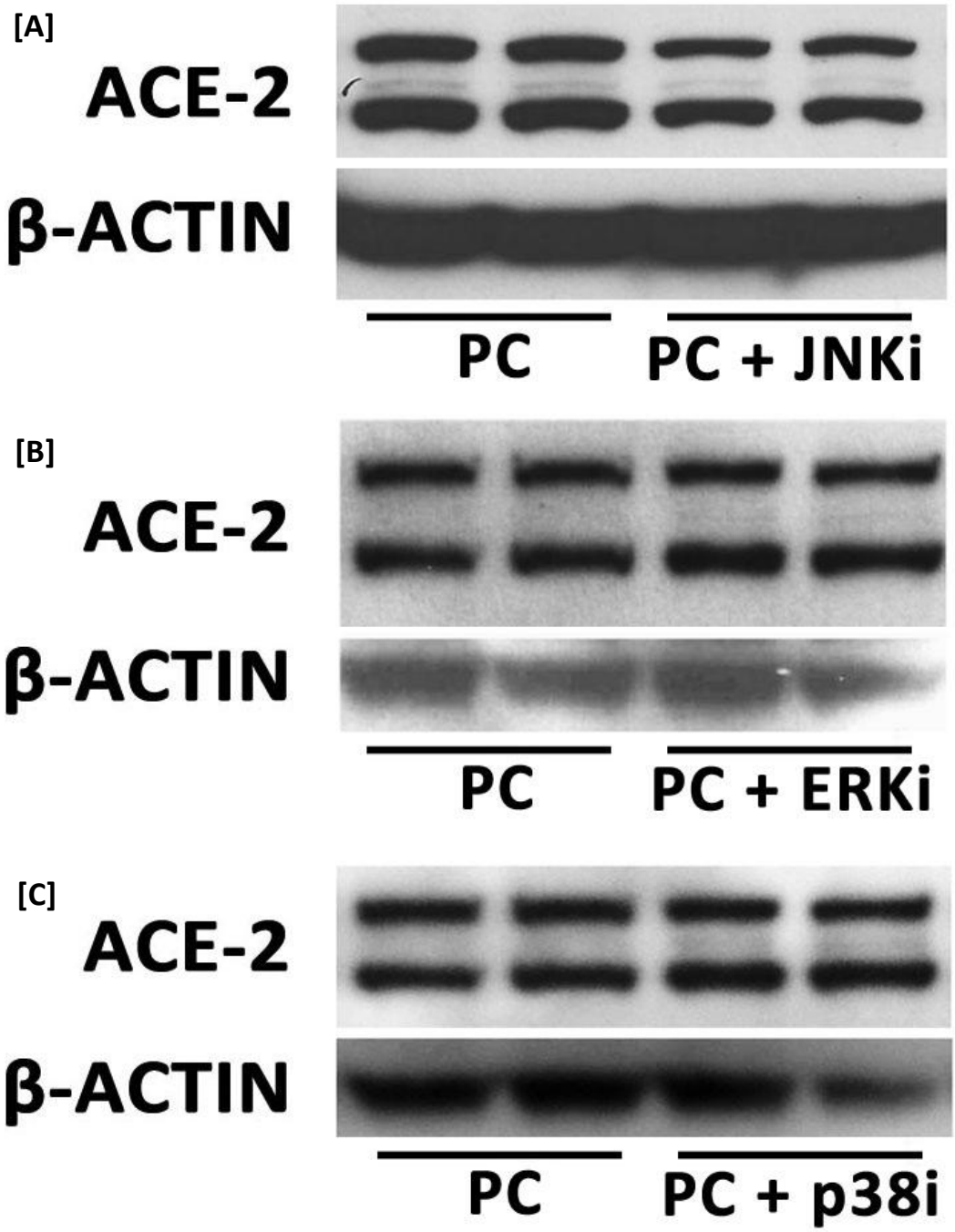

Figure 5.8. An inhibitor against JNK [A] blocked the up-regulation of ACE-2 in quiescent cells but inhibitors against ERK [B] and p38 [C] did not.

Discussion.

ACE-2 is a protective enzyme that is down-regulated in both human and experimental lung fibrosis.[4] This enzyme degrades the profibrotic peptide, ANGII into the anti-fibrotic peptide, ANG1-7. The regulation of ACE-2 has not been well elucidated. Various stimuli have been shown to down-regulate ACE-2. For instance, in cardiac myocytes and fibroblasts, ANGII or endothelin (ET-1) decreased both ACE-2 mRNA and protein.[27] These effects were abrogated with inhibitors of MAPK-1, suggesting the involvement of ERKs in regulating ACE-2. In AECs, our lab discovered that inducers of ER stress such as proteosome inhibitors, MG-132 or clasto-lactacystin β-lactone, and the G100S SP-C mutation, resulted in the down-regulation of ACE-2.[28] Additionally, the use of TAPI-2, an inhibitor of ADAM17/TACE, restored ACE-2 levels in the presence of these ER-stress inducers.[28] This suggests a role of ACE-2 ectodomain shedding as a mechanism in down-regulating this protective enzyme with these stimuli.

This study suggests another mechanism by which ACE-2 is down-regulated. In proliferating AECs, ACE-2 mRNA, protein, and enzymatic activity were reduced compared to cells in the quiescent non-proliferating state.[7] These data support the hypothesis that cell cycle state regulates ACE-2 expression. Moreover, the transition from a proliferating to a quiescent cell cycle state is through a JNK-mediated mechanism. Currently, our laboratory is attempting to elucidate the signaling pathways that are involved in this process.

A Preliminary Investigation on the Effects of TGF-β_1 on the ANG System in Pulmonary Fibroblasts

Introduction.

TGF-β_1 is a profibrotic cytokine that is implicated in the pathogenesis of IPF due to its ability to: 1) induce the apoptosis of AECs, 2) generate myofibroblasts from resident fibroblasts or through EMT, and 3) stimulate the production of extra-cellular matrix proteins.[29] These three events contribute to the dysregulated wound healing that is observed in IPF. Previous studies by our laboratory demonstrated that TGF-β_1 can induce the transcription of *AGT* in human pulmonary fibroblasts.[30] AGT is the only known precursor to the profibrotic peptide, ANGII. In IPF, both the mRNA and protein of AGT and TGF-β_1 are up-regulated, as well as the ANGII peptide.[31] The relationship between AGT and TGF-β_1 occurs through an autocrine cross-talk in myofibroblasts.[32] In addition to up-regulating *AGT*, I wanted to investigate if other components in the ANG system are affected by TGF-β_1.

Materials and Methods.

Cell Culture. The human fibroblast cell line, IMR-90 (ATCC, Manassas, VA) was cultured in MEM media supplemented with 10% FBS and 1% penicillin/streptomycin on 150 mm collagen I-coated plates. Prior to treatment with TGF-β_1, cells were washed 3x in serum-free MEM media before being serum-starved for 24 hours. IMR-90s in serum-free media were treated with TGF-β_1 (at a final concentration of 2 ng/mL) for 24 hours. At the end of the treatment period, the media was gently aspirated and cells were washed 1x in ice-cold PBS

before harvesting. Cytosolic and nuclear extracts were harvested from the same samples using the protocol published by Wu.[33]

Western Blotting. The protein concentration of the cytosolic fraction of the lysates were quantitated using the BCA Assay (ThermoScientific). 40 μg of each denatured sample was ran on 10% Tris-HCl polyacrylamide gels (Bio-Rad) at 120 V. This was followed by a transfer to PVDF membranes for 90 minutes at 100 V. Membranes were washed 3x in TBST before being blocked in 5% non-fat dry milk in TBST for 60 minutes at room temperature. Membranes were incubated with primary antibodies at 4°C overnight. These antibodies used were against ACE-2 at 1:2,000 dilution (Abcam, Eugene, OR), cathepsin D at 1:4,000 dilution (Santa Cruz Technology, Santa Cruz, CA), α-SMA-FITC at 1:1,000 dilution (Sigma-Aldrich, St. Louis, MO) and β-actin at 1:3,000 (Cell Signaling Technology, Beverly, MA). After overnight incubation, membranes were washed 4x in TBST buffer before incubation with secondary or tertiary antibodies: α-rabbit-HRP at 1:12,000, α-goat at 1:96,000, α-mouse at 1:5,000, or α-FITC at 1:10,000 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized by ECL detection systems (ThermoScientific, Rockford, IL). Densitometry was used to quantitate the bands using ImageJ (NIH).

Results.

TGF-β_1 Increases α-SMA. It is well known that TGF-β_1 induces the transition of fibroblasts into myofibroblasts. During this transition, they begin expressing α-SMA (**Figure 5.9**). Therefore, plates of IMR-90s that are treated with TGF-β_1 can be used to model myofibroblastic foci.

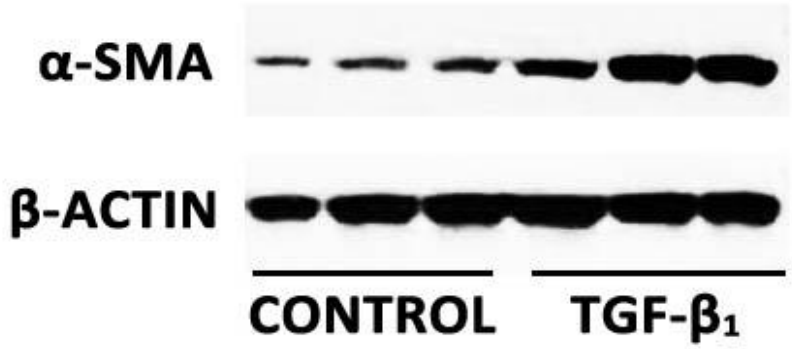

Figure 5.9. TGF-β_1 increases α-SMA, a marker for myofibroblasts.

Effects of TGF-β_1 on the ANG System. As published in a prior paper, TGF-β_1 significantly induces *AGT* transcription.[30] This effect was reproducible in a new set of IMR-90s (**Figure 3.4** in **Chapter 3**). Cathepsin D, an enzyme that cleaves AGT to form ANGI, the precursor to ANGII, is significantly up-regulated with TGF-β_1 [($p = 0.0024$ (**Figure 5.10A**)]. However, the ANGII degrading enzyme, ACE-2 was significantly reduced in IMR-90s treated with TGF-β_1 compared to controls [$p = 0.0015$ (**Figure 5.10B** and **Figure 5.10C**)]. This suggests that TGF-β_1 creates an imbalance in the ANG system by up-regulating the ANGII forming axis (AGT and cathepsin D) and down-regulating the ANGII degrading axis (ACE-2).

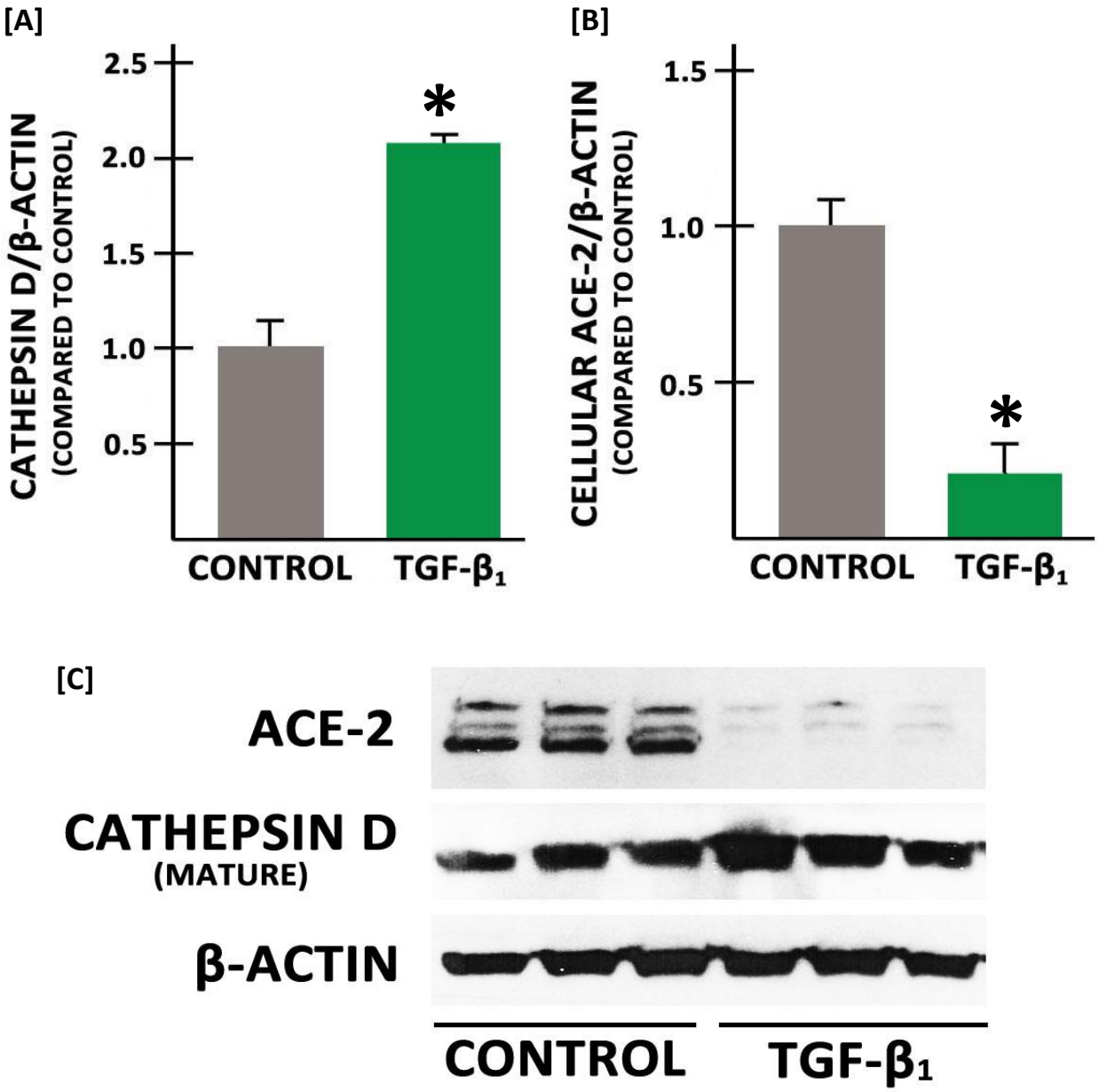

Figure 5.10. Effects of TGF-β_1 on cathepsin D and ACE-2. [A] TGF-β_1 significantly increases cathepsin D protein to more than 2-fold ($p = 0.0024$) and **[B]** reduces cellular ACE-2 protein more than 4-fold ($p = 0.0015$). **[C]** A western blot that is representative of the quantitated results. Bars are mean $\pm$ SEM with $n = 3$.

Future Studies.

The preliminary data presented here suggests that TGF-β_1 up-regulates the ANGII producing arm and down-regulates the protective ANGII degrading arm of the ANG system. This imbalance will favor the generation of the profibrotic peptide ANGII. Therapeutically, the use of ANG1-7, ACE-2, or ARBs can ideally bring the ANG system back into balance.

The increase in both *AGT* transcription and cathepsin D protein and the decrease in cellular ACE-2 protein are predicted to increase ANGII. AGT and cathepsin D are part of the rate-limiting step in the generation of ANGII. Therefore, alterations in both components will push the reaction to favor the production of ANGII. As a consequence of the rapid conversion of AGT into ANGII, the protein form of AGT was difficult to visualize on a Western blot. In order to circumvent this, future studies will utilize Pepstatin A, a potent inhibitor of aspartyl proteases, such as cathepsin D. Additionally, we would like to determine if the alterations in cathepsin D and ACE-2 are regulated on a transcriptional level like *AGT* and if so, what TFs mediate this process. It will also be interesting to see if ACE-2 ectodomain shedding or a JNK-mediated mechanism is involved in the TGF β_1-mediated decrease of ACE-2. Lastly, we would like to determine if the myofibroblast phenotype is reversible with manipulation of the ANG system with ACE-2, ANG1-7, or ARBs. If so, it will provide support for the use of manipulators of the ANG system as therapeutic drugs in treating IPF. This is especially true for ARBs, as these drugs are widely use with well-known therapeutic profiles in treating hypertension that can be extrapolated to IPF.

APPENDIX

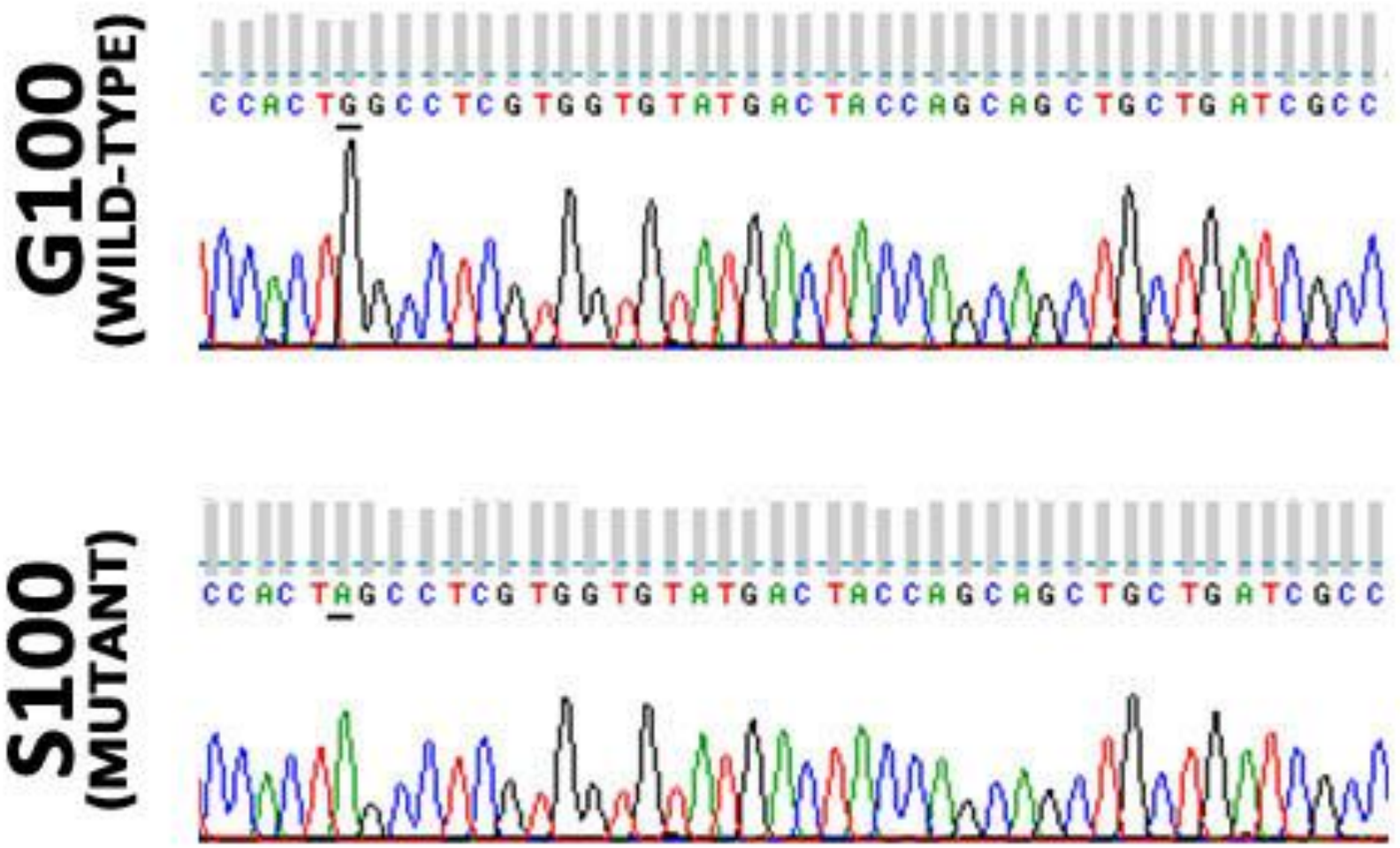

Figure S3. Chromat tracings reveal the G100S mutation caused by a G to A SNP.

REFERENCES

REFERENCES

1. Wang, R., Ramons, C., Joshi, I., Zagariya, A., Pardo A., Selman, M., and Uhal, B.D. Human lung myofibroblast derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides. *Am. J. Physiol.* 277:L1158-L1164 (1999).

2. Li, X., Molina-Molina, M., Abdul-Hafez, A., Ramirez, J., Serrano-Mollar, A., Xaubet, A., and Uhal, B.D. Extravascular sources of lung angiotensin peptide synthesis in idiopathic pulmonary fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* 291:L887-L895.

3. Kasper, M., Lackie, P., Haase, M., Schuh, D, and Müller, M. Immunolocalization of cathepsin D in pneumocytes of normal human lung and in pulmonary fibrosis. *Virchows Archiv.* 428:207-215 (1996).

4. Li, X., Molina-Molina, M., Abdul-Hafez, A., Uhal, V., Xaubet, A. and Uhal, B.D. Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis. *Am. J. Physiol. Cell Mol. Physiol.* 295:L178-L185 (2008).

5. Huentelman, M.J., Grobe, J.L., Vazquez, J., Steward, J.M., Mecca, A.P., Katovich, M.J., Ferrario, C.M., and Raizada, M.K. Protection from angiotensin-II induced cardiac hypertrophy and fibrosis by systemic lentiviral delivery of ACE2 in rats. *Exp. Physiol.* 90:783-790 (2005).

6. Liu, Z., Huang, X.R., Chen, H.Y., Penninger, J.M., and Lan, H.Y. Loss of angiotensin-converting enzyme 2 enhances TGF-β/Smad-mediated renal fibrosis and NF-κB-driven renal inflammation in a mouse model of obstructive nephropathy. *Lab. Invest.* 92:650-661 (2012).

7. Uhal, B.D., Dang, M.T., Dang, V., Llatos, R., Cano, E., Abdul-Hafez, A., Markey, J., Piasecki, C.C., and Molina-Molina, M. Cell cycle dependence of ACE-2 explains downregulation in idiopathic pulmonary fibrosis. *Eur. Respir. J.* 42:198-210 (2013).

8. Wang, R., Zagariya, A., Ang, E., Ibarra-Sunga, O., and Uhal, B.D. Fas-induced apoptosis of alveolar epithelial cells requires angiotensin II generation and receptor interaction. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 277:L1245-L1250 (1999).

9. Li, X., Rayford, H., and Uhal, B.D. Essential roles for angiotensin receptor AT1a in bleomycin-induced apoptosis and lung fibrosis in mice. *Am. J. Pathol.* 163:2523-2530 (2003).

10. Bargout, R., Jankov, A., Dincer, E., Wang, R., Komodromos, T., Ibarra-Sunga, O., Filippatos, G., and Uhal, B.D. Amiodarone induces apoptosis of human and rat alveolar epithelial cells in vitro. *Am. J. Physiol.* 278:L1039-L1044 (2000).

11. Li, X., Zhang, H., Soledad-Conrad, V., Zhuang, J., and Uhal, B.D. Bleomycin-induced apoptosis of alveolar epithelial cells requires angiotensin synthesis *de novo. Am. J. Physiol. Lung Cell. Mol. Physiol.* 284:L501-L507 (2003).

12. Uhal, B.D., and Nguyen, H. The Witschi Hypothesis revisited after 35 years: genetic proof from SP-C BRICHOS domain mutations. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 305:L906-911 (2013).

13. Ono, S., Tanaka, T., Ishida, M., Kinoshita, A., Fukuoka, J., Takaki, Mi., Sakamoto, N., Ishimatsu, Y., Kohno, S., Hayashi, T., Senba, M., Yasunami, M., Kubo, Y., Yoshida, L.M., Kubo, H., Ariyoshi, K, Yoshiura, K., and Morimoto, K. Surfactant protein C G100S mutation causes familial pulmonary fibrosis in Japanese kindred. *Eur. Respir. J.* 38:861-869 (2001).

14. Li, X., Rayford, H., Shu, R., Zhuang, J., and Uhal, B.D. Essential role for cathepsin D in bleomycin-induced apoptosis of alveolar epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 284:L501-L507 (2003).

15. Maguire, J.A., Mulugeta, S., and Beers, M.F. Multiple ways to die: Delineation of the unfolded protein response and apoptosis induced by surfactant protein C BRICHOS mutants. *Int. J. Biochem. Cell Biol.* 44:101-12 (2012).

16. Wang, W.J., Mulugeta, S., Russo, S.J., and Beers, M.F. Deletion of exon 4 from human surfactant protein C results in aggresome formation and generation of a dominant negative. *J. Cell Science.* 116:683-692 (2003).

17. Lawson, W.E., Crossno, P.F., Polosukhin, V.V., Roldan, J., Cheng, D.S., Lane, K.B., Blackwell, T.R., Xu, C., Markin, C., Ware, L.B., Miller, G.G., Loyd, J.E., and Blackwell, T.S. Endoplasmic reticulum stress in alveolar epithelial cells is prominent in IPF: association with altered surfactant protein processing and herpesvirus infection. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 294:L1119-L1126 (2008).

18. Johansson, H., Nerelius, C., Nordling, K., and Johansson, J. Preventing amyloid formation by catching unfolded transmembrane signals. *J. Mol. Biol.* 389:227-229 (2009).

19. Nogee, L.M., Dunbar, A.E. III, Wert, S., Askin, F., Hamvas, A., and Whitsett, J.A. Mutations in the surfactant protein C gene associated with familial interstitial lung disease. *Chest.* 121:20S-21S (2002).

20. Guillot, L., Epaud, R., Thouvenin, G., Jonard, L., Mohsni, A., Couderc, R., Counil, F., de Blick, J., Taam, R.A. Le Bourgeois, M., Reix, P., Flamein, F., Celment, A., and Feldmann, D. New surfactant protein C gene mutations associated with diffuse lung disease. *J. Med. Genet.* 46:490-494 (2009).

21. Kuwano, K., Kunitake, R., Maeyama, T., Hagimoto, N., Kawasaki, M., Matsuba, T., Yoshimi, M., Inoshima, I., Yohsida, K., and Hara, N. Attenuation of bleomycin-induced pneumopathy in mice by a caspase inhibitor. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 280:L316-L325 (2001).

22. Budinger, G.R., Mutlu, G.M., Eisenbart, J., Fuller, A.C., Bellmeyer, A.A., Baker, C.M., Wilson, M., Ridge, K., Barrett, T.A. Lee, V.Y., and Chandel, N.S. Proapoptotic Bid is required for pulmonary fibrosis. *Proc. Natl. Acad. Sci. USA.* 103:4604-4609 (2006).

23. Wang, R., Ibarra-Sunga, O., Pick, R., and Uhal, B.D. Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. *Am. J. Physiol. Lung Cell Mol. Physiol.* 279:L143-L151 (2000).

24. Patel, V.B., Clarke, N., Wang, Z., Fan, D., Parajuli, N., Basu, R., Putko, B., Kassiri, Z., Turner, A.J., and Oudit, G.Y. Angiotensin II induced proteolytic cleavage of myocardial ACE2 is mediated by TACE/ADAM-17: a positive feedback mechanism in the RAS. *J. Mol. Cell. Cardiol.* 66:167-176 (2014).

25. Epelman, S., Tang, W.H., Chen, S.Y., Van Lente, F., Francis, G.S., and Sen, S. Detection of soluble angiotensin-converting enzyme 2 in heart failure: insights into the endogenous counter-regulatory pathway of the renin-angiotensin-aldosterone system. *J. Am. Coll. Cardiol.* 52:750-754 (2008).

26. Uhal, B.D., Li, X., Xue, A., and Abdul-Hafez, A. Regulation of alveolar epithelial cell survival by the ACE-2/angiotensin1-7/Mas axis. *Am. J. Physiol. Lung Cell Mol. Physiol.* 301:L269-L274 (2011).

27. Gallagher, P.E., Ferrario, C.M., and Tallant, E.A. Regulation of ACE2 in cardiac myocytes and fibroblasts. *Am. J. Physiol. Heart Circ. Physiol.* 295:H2373-H2379 (2008).

28. Uhal, B.D., Nguyen, H., Dang, M.T., Gopallawa, I., Jiang, J., Dang, V., Ono, S., and Morimoto, K. Abrogation of ER stress-induced apoptosis of alveolar epithelial cells by angiotensin 1-7. *Am. J. Physiol. Lung Cell Mol. Physiol.* 305:L33-L41 (2013).

29. Fernandez, I., and Eickelberg, O. The impact of TGF-β on lung fibrosis. *Proc. Am. Thorac. Soc.* 9(3):111-116 (2012).

30. Abdul-Hafez, A., Shu, R., and Uhal, B.D. JunD and HIF-1alpha mediate transcriptional activation of angiotensinogen by TGF-beta1 in human lung fibroblasts. *FASEB J.* 23:1655-1662 (2009).

31. Uhal, B.D., Li, X., Piasecki, C., and Molina-Molina, M. Angiotensin signaling in pulmonary fibrosis. *Int. J. Biochem. Cell Biol.* doi:10.1016/i.biocel.2011.11.019 (2011).

32. Uhal, B.D., Kim, J.K., Li, X., and Molina-Molina, M. Angiotensin-TGF-β1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages. *Curr. Pharm. Des.* 13:1247-1256 (2007).

33. Wu, K.K. Analysis of protein-DNA binding by streptavidin-agarose pulldown from *Methods in Molecular Biology vol. 338: Gene Mapping, Discovery, and Expression: Methods and Protocols.* Edited by Bina, M. 281-290 (Humana Press Inc., Totowa, New Jersey USA 2006).

CHAPTER 6

A SUMMARY AND CONCLUSION: TRANSLATIONAL IMPLICATIONS OF THE ANG SYSTEM IN IPF

Research Significance

At the recent 2012 International Colloquium on Lung and Airway Fibrosis in Modena, Italy, experts in the field recognized the need to identify phenotypes in order to sub-classify IPF, which is designated as an orphan disease. An orphan disease is defined as either a disease affecting less than 200,000 people in the United States or a disease in which there is little incentive for pharmaceutical companies to develop new drugs. However, if any Food and Drug Administration (FDA)-approved drugs that are already on the market can be found to be effective, there would be no need to generate new drugs. This research study has the potential to generate biomarkers to identify a sub-class of IPF patients who are responsive to treatment with angiotensin receptor blockers (ARBs), ACE-2, and ANG1-7. Drugs in the first category are already approved by the FDA and are widely used to treat hypertension. A pilot study demonstrated that Losartan (an ARB) stabilized or improved lung function in IPF patients.[1] This research shows the promise of ARBs as treatments for IPF. The profiles of these drugs are well-known, thus allowing the drug to be easily implemented. Along similar lines, this same concept can be applied to other fibrotic diseases.

The ANG System in IPF

IPF is the most common form of interstitial lung disease with a prevalence of about 20 per 100,000.[2] It is a "chronic, progressive, and irreversible" condition with a bias towards males in their fifth to eighth decade of life.[2] Upon diagnosis, the mean survival is three years.

Currently, the only therapy to prolong survival is lung transplantation.[2] However, the five year post-operative survival rate is 44%.[2] The current standard of care for the treatment of IPF includes the use of corticosteroids and immunosuppressants - both of which have minimal benefits. The lack of benefit from these treatments demonstrates an incomplete understanding of the pathogenesis of IPF.

IPF is the result of abnormal wound healing consisting of persistent injury to AECs, aberrant fibroblast proliferation and the accumulation of extracellular matrix proteins.[3] Our laboratory has implicated a role for the ANG system in this process. Injured AECs transforms latent TGF-β_1 into its active form. Type II AECs are unable to replace the injured AECs and becomes hyperplastic. These hyperplastic AECs secrete TGF-β_1. TGF-β_1 induces profibrotic effects by: 1) mediating the transformation of fibroblasts into myofibroblasts, 2) increasing pro-fibrotic genes [such as collagen and alpha-smooth muscle actin (α-SMA)], 3) suppressing the apoptosis of fibroblasts, 4) inducing the apoptosis of AECs, and 5) inducing *AGT* transcription in fibroblasts to generate the profibrotic peptide, ANGII.[4-6] ANGII induces the apoptosis of AECs, recapitulating the repetitive injury of the alveolar epithelium. These injured AECs contribute to the aberrant fibroblast proliferation by activating TGF-β_1.

From these data, it is hypothesized that variants in *AGT*, the only known precursor to the profibrotic peptide, ANGII, and *TGF-β_1* can serve as biomarkers for IPF by predicting worse pulmonary function. In our studies, we looked at variants that were associated with changes in the levels of *AGT* and TGF-β_1. For *AGT,* the -20 and -6 SNPs were shown to affect the

transcription rate in hepatocytes.[7-8] Whereas variants in codon 10 and 25 in TGF-β_1 affected its rate of secretion.[9] In our studies, the CC genotype at -20, the AA genotype at -6, and the CA haplotype at -20 and -6 respectively, were significantly associated with reduced diffusing capacity in IPF cohorts from the United States and Spain.[10] Additionally, we also observed that the CA haplotype had about a 1.5-fold higher rate of *AGT* transcription compared to the AG haplotype in human fibroblasts. For the *TGF-β_1* variant, the presence of the Proline/Proline genotype at codon 10 was associated with a reduction in diffusing capacity in an IPF cohort from the United States. However, no associations were found for the codon 25 variant. Surprisingly, in both genes this was in a sex-dependent manner, reflecting the male bias that is observed in IPF. Due to the lack of sufficient numbers of patients containing both the *AGT* and TGF-β_1 "risk haplotype," we were unable to assess if the combination of these variants would predict worse pulmonary function (as measured by lower values for the diffusing capacity than observed with either variant alone). Additionally, our attempts to study the association of these variants in relation to HRCT data was limited by the small sample size (n = 65).

In addition to inducing *AGT* transcription, preliminary data indicates that TGF-β_1 can also up-regulate cathepsin D and down-regulate the protective enzyme, ACE-2 in pulmonary fibroblasts. The increase in *AGT* and cathepsin D along with the decrease in ACE-2 are hypothesize to result in high levels of ANGII as these imbalances favor the ANGII producing axis. In human AECs, induction of ER-stress (by proteosome inhibitors or the G100S SP-C mutation) and cell cycle state can also down-regulate ACE-2.[11-12] Data suggests that ER-stress reduction

of ACE-2 is mediated by ACE-2 ectodomain shedding whereas the regulation of ACE-2 by cell cycle state is JNK-mediated. Using these data, future studies will explore the roles of ACE-2 ectodomain shedding and JNK signaling as potential mechanisms for the down-regulation of ACE-2 mediated by TGF-β_1 in human pulmonary fibroblasts.

Potential of *AGT* and TGF-β_1 Haplotypes as IPF Biomarkers

In IPF, both AGT and TGF-β_1 mRNA and protein, as well as ANGII are up-regulated, whereas ACE-2 is down-regulated.[3, 13] This suggests an imbalance in the ANG system where the ANGII generating axis is favored over the ANGII degrading axis. In human pulmonary fibroblasts, TGF-β_1 induces the transcription of *AGT* and preliminary data indicates that it also up-regulates cathepsin D and down-regulates ACE-2. Cathepsin D and AGT are part of the rate-limiting step in the generation of ANGII whereas ACE-2 degrades ANGII into the anti-fibrotic peptide, ANG1-7. Moreover, the presence of the CA haplotype at the -20 and -6 positions in *AGT* resulted in higher rates of TGF-β_1-inducible *AGT* transcription compared to the AG haplotype in pulmonary fibroblasts. In IPF cohorts, variants in *AGT* at the -20 and -6 positions and at codon 10 in *TGF-β_1* predicted lower diffusing capacity that was unique to males. The lower diffusing capacity is hypothesized to be correlated with greater severity of the disease. Since IPF is a restrictive type of lung disease, lower diffusing capacity reflects lower rates of oxygen across the alveolar-capillary membrane due to its thickened architecture. Additionally, compared to the FVC, the diffusing capacity is a more sensitive predictor of this gas exchange

and a more reliable predictor of survival in IPF.[14] The FVC is the maximum volume that is rapidly and forcibly expired during a spirometry test. Factors that limit chest expansion, such as kyphosis or scoliosis, rib fractures, compression of the spine, respiratory muscle weakness, and changes in lung compliance, can influence the FVC. All of these can be altered in a variety of disease state that are not specific to the pulmonary system.[15] Parenchymal lung diseases have direct effects on the rate of transfer of oxygen across the alveolar-capillary membrane due to its influence on the thickness and the surface area of the membrane.[16] This relationship reflects what is observed in IPF, where the accumulation (or decrease degradation) of extra-cellular matrix proteins in the interstitium results in a thickened membrane, thereby decreasing the rate of transfer of oxygen across the membrane. Likewise, repetitive injury to the alveolar epithelium with concomitant apoptosis of AECs will decrease the surface area resulting in lower rates of oxygen transfer.

Although the results of this study does not predict the risk of having IPF, it does suggest that variants in *AGT* and *TGF-β_1* can be genetic modifiers in the severity of IPF. Thereby supporting a role for these variants as potential biomarkers for IPF and that targeting the ANG system can be a therapeutic approach for treating this disease. The presence of the CC genotype at -20, AA genotype at -6, CA haplotype at -20 and -6 respectively in *AGT* and/or the *TGF-β_1* codon 10 Proline/Proline (CC) variant are predicted to favor the production of ANGII, thereby promoting the fibrotic response in IPF (**Figure 6.1**) . As a biomarker, it will allow physicians to assess the response to treatments with manipulators of the ANG system

including, ARBs, ACE-2, and ANG1-7. Promising support for this idea stems from a small pilot study where Losartan, an ARB, stabilized or improved lung function in IPF patients.[1] The wide-spread use of ARBs with its safe therapeutic profile in treating hypertension will make it easier to implement in larger clinical trials for IPF. In the future, personalized medicine will be tailored to fit an individual and a part of this niche can be filled with one's haplotype.

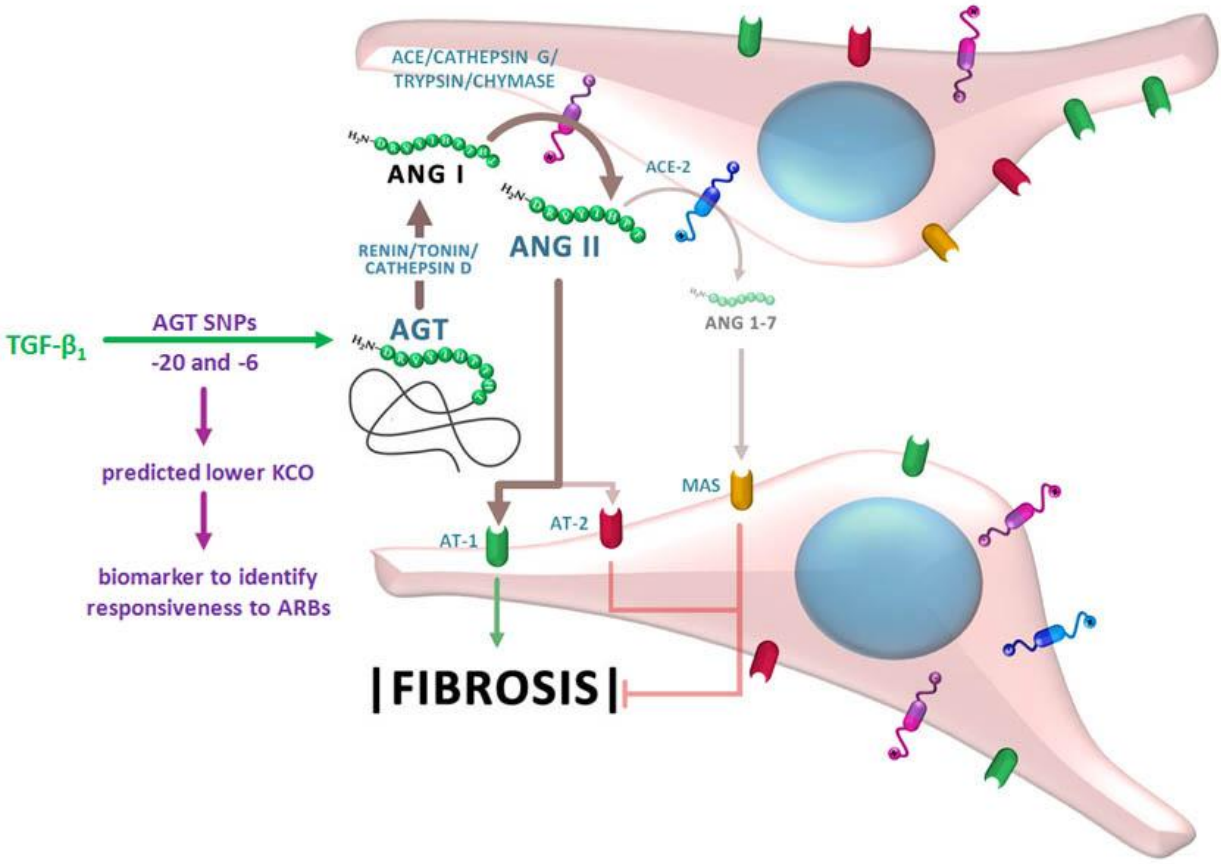

Figure 6.1. Summary of the effects of *AGT* and TGF-β_1 SNPs in IPF.

TGF-β_1 = transforming growth factor beta; AGT = angiotensinogen; KCO = diffusion capacity for carbon monoxide; ARBs = angiotensin receptor blockers; ANG = angiotensin; AT = angiotensin receptor type; ACE = angiotensin converting enzyme

REFERENCES

REFERENCES

1. Couluris, M., Kinder, B.W., Xu, P., Gross-King, M., Krischer, J., and Panos, R.J. Treatment of idiopathic pulmonary fibrosis with losartan: a pilot project. *Lung.* 190:523-527 (2012).

2. King, T.E. Jr., Pardo, A., and Selman, M. Idiopathic pulmonary fibrosis. *Lancet.* doi:10.1016/20140-6736(11)60052-4 (2011).

3. Uhal, B.D., Li, X., Piasecki, C., and Molina-Molina, M. Angiotensin signaling in pulmonary fibrosis. *Int. J. Biochem. Cell Biol.* doi:10.1016/i.biocel.2011.11.019 (2011).

4. Uhal, B.D., Kim, J.K., Li, X., and Molina-Molina, M. Angiotensin-TGF-β1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages. *Curr. Pharm. Des.* 13:1247-1256 (2007).

5. Selman, M., King, T.E., and Pardo, A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann. Intern. Med.* 134:136-151.

6. Fernandez, I., and Eickelberg, O. The impact of TGF-β on lung fibrosis. *Proc. Am. Thorac. Soc.* 9:111-116 (2012).

7. Yanai, K., Saito, T., Hirota, K., Kobayashi, H., Murakami, K., and Fukamizu, A. Molecular variation of the human angiotensinogen core promoter element located between the TATA box and transcription initiation site affects its transcriptional activity. *J. Biol. Chem.* 272:30558–30562 (1997).

8. Inoue, I., Nakajima, T., Williams, C.S., Quackenbush, J., Puryear, R., Powers, M., Cheng, T., Ludwig, E.H., Sharma, A.M., Hata, A., Jeunemaitre, X., and Lalouel, J.M. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. *J. Clin. Invest.* 99:1786-1797 (1997).

9. Grainger, D.J., Heathcote, K., Chiano, M., Snieder, H., Kemp, P.R., Metcalfe, J.C., Carter, N.D., and Spector, T.D. Genetic control of the circulating concentration of transforming growth factor type beta1. *Hum. Mol. Genet.* 8:93-97 (1999).

10. Dang, M.T., Gu, C., Klavanian, J.I., Jernigan, K.A., Friderici, K.H., Cui, Y., Molina-Molina, M., Ancochea, J., Xaubet, A., and Uhal, B.D. Angiotensinogen promoter polymorphisms predict low diffusing capacity in U.S. and Spanish IPF cohorts. *Lung.* 191:353-360 (2013).

11. Uhal, B.D., Nguyen, H., Dang, M.T., Gopallawa, I., Jiang, J., Dang, V., Ono, S., and Morimoto, K. Abrogation of ER stress-induced apoptosis of alveolar epithelial cells by angiotensin 1-7. *Am. J. Physiol. Lung Cell Mol. Physiol.* 305:L33-L41 (2013).

12. Uhal, B.D., Dang, M.T., Dang, V., Llatos, R., Cano, E., Abdul-Hafez, A., Markey, J., Piasecki, C.C., and Molina-Molina, M. Cell cycle dependence of ACE-2 explains downregulation in idiopathic pulmonary fibrosis. *Eur. Respir. J.* 42:198-210 (2013).

13. Li, X., Molina-Molina, M., Abdul-Hafez, A., Uhal, V., Xaubet, A. and Uhal, B.D. Angiotensin converting enzyme-2 is protective but downregulated in human and experimental lung fibrosis. *Am. J. Physiol. Cell Mol. Physiol.* 295:L178-L185 (2008).

14. Raghu, G., Collard, H.R., Egan, J.J., Fernando, J.M., Behr, J., Brown, K.K., Colby, T.V., Cordier, J.F., Flaherty, K.R., Lasky, J.A., Lynch, D.A., Ryu, J.H., Swigris, J.J., Wells, A.U., Ancochea, J., Bouros, D., Carvalho, C., Costabel, U., Ebina, M., Hansell, D.M., Johkoh, T., Kim, D.S., King, T.E., Kondoh, Y., Myers, J., Müller, N.L., Nicholson, A.G., Richeldi, L., Selman, M., Dudden, R.F., Griss, B.S., Protzko, S.L., and Schünemann, H.J. An official ATS/ERS/JRS/ALAT statement: Idiopathic pulmonary fibrosis: Evidence-based guidelines for diagnosis and management. *Am. J. Respir. Crit. Care Med.* 183:788-824 (2011).

15. Petty, T.L., and Enright, P.L. Simple office spirometry for primary care practitioners. *National Lung Health Education Program* (2003). http://www.nlhep.org/Documents/simple_office_spirometry.pdf

16. Grippi, M.A. Chapter 9: Gas Exchange in the Lung in *Lippincott's Pathophysiology Series: Pulmonary Pathophysiology.* (J.B. Lippincott Company, Philadephia, PA, 1995).