Transcriptional Regulation of Angiotensinogen by Oncogenic K-Ras

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Abstract

As we age, our cells undergo irreversible growth arrest known as cellular senescence. Cells can undergo replicative senescence, which is caused by telomere shortening, or they can undergo stress-induced premature senescence (SIPS) which can be caused by oncogenic activation, DNA damage, cytotoxic drugs, and oxidative stress. Since senescent cells cannot replicate, cellular senescence is a powerful tumor suppressor mechanism. Lung cancer is the most common cancer worldwide and a continued public health issue. Despite public health efforts to reduce lung cancer cases by informing the public of the dangers of smoking, it remains a major public health concern.

Oncogenic K-Ras promotes senescence in normal cells but fuels transformation of cancer cells after the senescence barrier is bypassed. The mechanisms regulating this pleiotropic function of K-Ras remain to be fully established and bear high pathological significance. Angiotensinogen (AGT) is the precursor to Angiotensin II, a main regulator of systemic blood pressure. Our laboratory has novel findings showing that Ang II induces senescence in normal cells but boosts transformation in lung cancer cells. Here, we tested the hypothesis that oncogenic K-Ras activates the AGT gene promoter both in normal and lung cancer cells through different molecular mechanisms. We find that K-Ras^{G12V} activates the AGT promoter in normal cells through the transcription factor KLF6. We also show that AGT protein expression is elevated in non-small cell lung cancer cells expressing K-Ras^{G12V} and that the transcription factor HMGA1 activates the AGT promoter in these cells. STAT3 signaling is activated in lung cancer. We find that

downregulation of HMGA1 inhibits STAT3 activation in lung cancer cells. Taken together, these results indicate that KLF6 and HMGA1 are key transcription factors through which oncogenic K-Ras activates the AGT/Ang II pathway in normal and lung cancer cells, respectively. Thus, KLF6 and HMGA1 represent therapeutic targets that can be exploited to develop novel anticancer interventions aimed at boosting oncogene-induced senescence in normal cells while inhibiting cell transformation of lung cancer cells.

Table of Contents

List of Tables

List of Figures

1.0 Background and Significance

1.1 Aging

Aging is a natural part of life which is associated with an increase in many degenerative diseases and cancer. The exact mechanism of aging is unclear, but there are many theories as to how and why we age. One theory is the free radical theory of aging. This theory states that as we get older, production of superoxide radicals increases due to dysfunction of the mitochondrial electron transport chain. These radicals build up and cause damage to cellular elements such as structural proteins. This damage causes a rapid decrease in functionality of the cell. Another theory is the senescence theory of aging which states that accumulation of senescent cells directly contributes to the aging process and the development of age-related diseases due to the senescent cell's loss of replication property and the ability of senescent cells to secrete cytokines (Newgard & Sharpless, 2013). Many organs require the continued replication of cells to maintain their functionality. As we age, senescent cells accumulate in these organs and negatively affect organ function due to their inability to replace dead cells (Volente et al., 2015). Along with the loss of a cell's replicating ability, senescent cells also secrete cytokines that can recruit immune cells and cause inflammation or induce neighboring cells to undergo senescence or apoptosis. Chronic inflammation is a defining characteristic of aging in humans and other mammals and contributes to many age-related phenotypes (Newgard & Sharpless, 2013).

1.2 Introduction to Cellular Senescence

Our cells acquire damage daily. As we age, this damage accumulates and can lead to a decline in cell and tissue function as well as an increase in the development of hyperplasia (Campisi, 2013). Our cells can respond to this damage by either apoptosis, autophagy, or senescence. Different cell types undergo different responses, and damaged epithelial cells and fibroblasts tend to undergo senescence (Dodig et al., 2019). There are two types of senescence: replicative senescence and stress-induced premature senescence (SIPS). Replicative senescence is dependent on the number of cell divisions and is controlled by telomere shortening. SIPS can be caused by oncogene activation, DNA damage, cytotoxic drugs, and oxidative stress (Volonte et al., 2015).

Senescent cells have unique characteristics that make them identifiable. Senescent cells are arrested in the G1 phase of the cell cycle. They can no longer replicate, but they can still perform regular cell functions. They also have increased p53 activity, increased p21 and p16 protein expression, and hypophosphorylation of retinoblastoma (Rb) and DDR proteins (Volonte et al., 2014). The cells morphology also changes to become large and flat, and they show increased β-galactosidase activity (Volonte et al., 2002).

Senescent cells can also develop a senescence-associated secretory phenotype (SASP) (Campisi, 2013) which causes the cell to release growth factors, cytokines, and proteases (Volonte et al., 2015). Typically, only cells that become senescent due to DNA damage, dysfunctional telomeres, and oxidative stress develop SASP. There are two theories as to why SASP evolved. One reason is because it allows senescent cells to communicate that they are damaged to their neighboring cells. Another reason is because it stimulates clearance of damaged cells by the immune system. Together, SASP and cellular senescence prevent cancer development and

promote tissue repair and healing. SASP may have roles other than preventing cancer due to its paracrine activities (Campisi, 2013). However, in cancer SASP can be pro-tumorigenic.

1.3 Cellular Senescence and Cancer

1.3.1 Cellular Senescence acts as a Tumor Suppressor

Senescence acts as a tumor suppressor mechanism. Cells with DNA damage that become senescent can no longer proliferate and pass on their damaged DNA. Certain SASP components also play a role in tumor suppression because they act in an autocrine fashion which provides strength in growth arrest. IL-6, IL-8, and IGFBP7 are some components released by SASP that reinforce growth arrest when cells become senescent due to oncogenic forms of RAS and BRAF. Senescent cells can also recruit the immune system to help with clearance of oncogene expressing premalignant and malignant cells (Campisi, 2013).

There are several lines of evidence that show that senescence suppresses tumorigenesis. Many tumors have been shown to contain cells that have either partially or completely overcome senescence. The senescence checkpoint is often disabled by oncogenes. p53 and pRB are required for the senescence response, but they are the two most commonly lost tumor suppressors. When p53 or pRB pathways are inactivated in the germline, cells cannot undergo senescence and organisms are cancer prone (Krtolica et al., 2001).

1.3.1.1 Oncogene Induced Senescence (OIS)

While oncogenic activation is critical for cancer development, it has also been shown to induce senescence which is referred to as oncogene induced senescence (OIS). OIS can be caused by the activation of K-Ras, B-Raf, AKT, E2F1, and cyclin E. It can also be caused by the inactivation of PTEN and NF1. OIS acts as a safety net against oncogenic stress (Liu et al., 2018).

In early studies, it was shown that activated Ras caused wild-type cells to undergo a short period of proliferation that was followed by irreversible growth arrest. These cells also had an accumulation of p53 and p16. This in vitro study was supported by the observation that senescence is only seen in benign tumors and not advanced tumors. There are several ways in which activation of oncogenes can cause senescence. One way is because of the accumulation of DNA damage. One model suggests that oncogene activation causes an accumulation of reactive oxygen species (ROS) which cause DNA damage. Another model suggests that DNA damage is caused by excessive replication due to the oncogenic signal. However, not all oncogenes induce DNA damage. Another way in which OIS can occur is through senescence-associated heterochromatic foci (SAHF). SAHF are caused by the recruitment of Rb and other proteins that change the chromatin structure. High mobility group A (HMGA) proteins are a component of SAHF. It has been shown that when these proteins are inactivated, along with p16, some cells can bypass OIS caused by Ras (Courtois-Cox et al., 2008).

OIS caused by activation of Ras is studied most since Ras mutations together with inactivation of p53 and p16 are the most prevalent mutations in human cancers. Oncogenic Ras fails to transform primary cells but can if the cells lack either p53 or p16. It has been suggested that cell cycle arrest caused by oncogenic Ras may cause a selective pressure to mutate p53 and

p16. In human pancreatic cancer, K-Ras amplification/mutation and loss of p16 and p53 are very common. In colorectal cancer, amplification/mutation of K-Ras occurs before p53 mutations (Serrano et al., 1997).

1.3.2 Cellular Senescence acts as a Tumor Enhancer

While it is well known that senescence and SASP play a role in preventing cancer, they are also implicated in age-related diseases including cancer. Through SASP, senescent cells can promote cell proliferation by releasing growth promoting proteins such as growth-related oncogenes (GROs) and vascular endothelial growth factor (VEGF). Senescent cells also promote inflammation via SASP. Inflammation plays a key role in aging and age-related degeneration (Campisi, 2013).

It has been shown that senescent cells can drive cancer pathology. When preneoplastic or neoplastic epithelial cells were grown on senescent fibroblasts, there was significant increase in growth compared to when they were grown on presenescent fibroblasts. However, genetically normal keratinocytes showed no significant difference in growth when grown on senescent vs. presenescent fibroblasts (Krtolica et al., 2001). These results were also seen in vivo when mice where co-injected with either preneoplastic or neoplastic epithelial cells and senescent or presenescent fibroblasts. The number and size of tumors was significantly higher in mice that were injected with senescent fibroblasts compared to presenescent fibroblasts. These mice also developed tumors earlier than the presenescent fibroblast mice (Krtolica et al., 2001).

In part, this increase in growth and number of tumors is due to both soluble and insoluble factors secreted by senescent cells such as MMP3 which promotes tumor cell invasion and VEGF which promotes tumor-driven angiogenesis (Campisi, 2013). The soluble and insoluble

factors released by senescent fibroblasts caused a significant increase in growth compared to the presenescent fibroblasts (Krtolica et al., 2001).

Other SASP factors that have been shown to promote malignancy are IL-6 and IL-8. These factors are released by senescent fibroblasts and cause premalignant epithelial cells to transition from epithelial to mesenchymal cells. After this transition, these cells can migrate and invade other tissues. Senescent cells caused by radiation and chemotherapeutic agents have been shown to release factors that protect neighboring tumor cells from being killed by those agents. Is SASP a cause of chemotherapeutic resistance (Campisi, 2013)?

As noted above, the immune system is stimulated by senescent cells and SASP. The immune system plays a role in clearing senescent cells. If this is the case, why do we have more senescent cells as we age? Some reasons for this include a less functional immune system as you age, higher frequency of senescent cells being produced, or immune evasion. SASP proteins include MMPs which can cleave cell surface ligands on natural killer (NK) target cells and the cell surface receptors of NK cells. This can thus prevent clearance of senescent cells by NK cells (Campisi, 2013).

If senescence has been implicated in aging and cancer, why did animals evolve such a mechanism? One possible explanation comes from the evolutionary theory of antagonistic pleiotropy which states that some genes are advantageous for young organisms but can have deleterious effects in aged organisms. It is likely that the deleterious effects of senescence are not seen in young organisms because senescent cells are rare, but as organisms age, the number of senescent cells increases and thus the unselected consequences are seen (Krtolica, 2001). Figure 2 shows the positive and negative effects senescent cells and SASP have in the body.

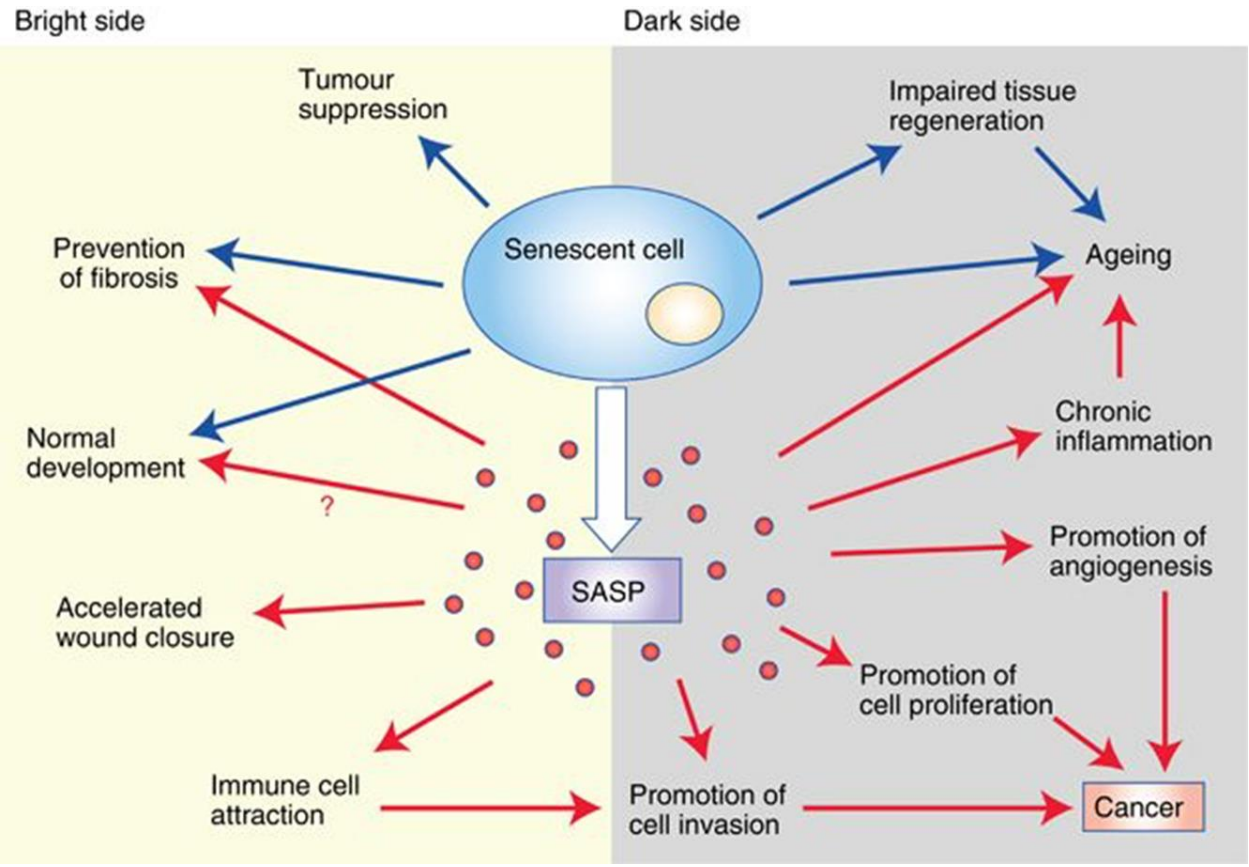

Figure 1. Advantages and Disadvantages of senescence and SASP

The blue arrows show the effects senescent cells have in the body. The red arrows show the effects the SASP has in the body. The left portion shows the advantages of both senescent cells and the SASP. The right portion shows the disadvantages of senescent cells and the SASP and how they ultimately lead to cancer

1.4 Angiotensinogen and Ang II

Angiotensinogen is the precursor for all angiotensin peptides. Angiotensinogen has 485 amino acids which includes a 33 amino acid signal peptide (Lu et al., 2016). It is mainly produced in the liver and then secreted into the circulation (Gallagher et al., 2011). Renin cleaves angiotensinogen to produce angiotensin I which is later cleaved by angiotensin converting

enzyme (ACE) to produce angiotensin II (Ang II) (Lu et al., 2016). ACE is primarily produced in endothelial cells of the lung (Gallagher et al., 2011). Ang II has been implicated in aging and cellular senescence. While Ang II is well known for its role in regulating blood pressure and renal electrolyte balance through the renin-angiotensin system, Ang II has been associated with the development of cardiovascular diseases due to its production of reactive oxygen species (ROS) and inducing SIPS (Herbert et al., 2008).

Herbert et. al. showed that vascular smooth muscle cells treated with Ang II for 24 hours had a significant increase in DNA damage compared to controls. When the cells could take up catalase prior to treatment, there was no significant increase in DNA damage. They also showed that the DNA damage caused by Ang II was mediated by the angiotensin II type 1 receptor (AT_1R). When they treated cells with E3174, an AT_1R antagonist, there was no DNA damage. Not only did this group show that Ang II is associated with increased DNA damage, but they also showed that Ang II treatment caused SIPS. In cells treated with Ang II, there was a significant increase in SA-β-gal activity, a hallmark of senescence. When cells were incubated with Ang II and either E3174 or catalase, SA-β-gal activity was comparable to the controls (Herbert et al., 2008).

Ang II causes superoxide (O_2^-) production by activating NAD(P)H oxidase when it binds to the AT_1R, and it uncouples endothelial NOS. Ang II also increases nitric oxide (NO) production which can react with O_2^- to increase ROS. In normal cells, the production of ROS by Ang II is tightly regulated. In conditions where the renin-angiotensin system is overactivated such as hypertension, diabetes, and ageing, Ang II-dependent oxidant generation plays a big role in tissue damage due to increased cell oxidation (de Cavanagh et al., 2011).

1.4.1 Local Ang II Signaling

Recent studies have shown high levels of Ang II in tissues such as the heart, brain, and lung compared to plasma levels. This suggests that tissue Ang II is locally synthesized. Many local systems are independent of renin and ACE but instead depend on other enzymes (Uhal et al., 2012).

The lung has two cell types that produce AGT: alveolar epithelial cells and myofibroblasts. It has been shown that AGT mRNA is upregulated in vivo after rat lungs are injected with bleomycin. Local angiotensin signaling in the lung must be activated for the pathogenesis of experimental lung fibrosis (Uhal et al., 2012).

1.4.2 Ang II Signaling in Cancer

Ang II has recently become a new target for cancer therapy due to its association with cell proliferation and migration, angiogenesis, inflammation, and extracellular matrix formation. Recent studies have shown the significance of Ang II in lung cancer. Neprilysin, which is a cell surface, zinc metalloprotease that cleaves peptide bonds and hydrolyzes Ang I to Ang (1-7), an endogenous, seven amino acid peptide hormone, is highly expressed in the lung. Cigarette smoke is known to inactivate neprilysin. Lung cancer cells transfected with a vector containing neprilysin showed a decrease in proliferation. This data suggests that the inability to degrade Ang II as well as a reduction in the production of Ang (1-7) may account for the aberrant growth of lung cancer cells since Ang (1-7) has been shown to have anti-proliferative properties (Gallagher et al., 2011).

In a retrospective study, the use of ACE inhibitors in patients was associated with significantly reduced relative risks of incident and fatal cancer. The inhibition of ACE decreases the conversion of Ang I to Ang II which indirectly causes an increase in Ang (1-7) production. When taken together with the data on neprilysin, reduction in cancer risk is not only associated with a decrease in Ang II levels but also from an increase in Ang (1-7) levels (Gallagher et al., 2011).

1.5 KLF6 and HMGA1 Transcription Factors

1.5.1 Krüppel-like factor 6 (KLF6)

Krüppel-like factor (KLF) proteins are transcription factors that bind the GC box of target genes. They can either up-regulate or down-regulate the expression of their target genes. KLF proteins have been shown to be associated with cell differentiation, development, growth, signal transduction, proliferation, and apoptosis. *KLF6* is a ubiquitously expressed transcription factor that has been suggested to be a tumor suppressor gene. *KLF6* has been shown to suppress growth and cause cell cycle arrest by interacting with different proteins. *KLF6* suppresses growth by upregulating expression of $p21^{WAF1/CIP1}$ in a p53-dependent manner. *KLF6* causes cell cycle arrest by interacting with cyclin D1 which disrupts complexes of cyclin D1 and cyclin-dependent kinase 4 (CDK4). Based on these interactions, it is likely that *KLF6* is a checkpoint protein that determines a cell's fate through the cell cycle (Ghaleb & Yang, 2008).

KLF6 is located at chromosome 10p15 which is where a lot of allelic loss is seen in non-small cell lung cancer (NSCLC). Previous studies have shown that *KLF6* is frequently

downregulated in NSCLCs. This downregulation was not due to any somatic mutation in the coding region of the gene. Most cases, but not all, showed a loss of heterozygosity (LOH). When NSCLCs exogenously expressed *KLF6*, these cells underwent apoptosis. These results taken together indicate that *KLF6* might be a tumor suppressor gene (Ito et al., 2004).

1.5.2 High mobility group A1 (HMGA1)

High mobility group A (HMGA) proteins are nonhistone chromatin remodeling proteins. These architectural transcription factors do not directly regulate gene expression but instead alter double-stranded DNA conformation by binding to the minor groove AT-rich regions. They can also interact with other transcription factors. The HMGA1 gene is highly expressed in embryonic and transformed cells but is almost undetectable in differentiated adult cells (Cleynen et al., 2007). *HMGA1* is a protooncogene (Pedulla et al., 2001), and high levels of HMGA1 have been reported to be associated with a highly malignant phenotype (Lin & Peng, 2016). However, it is unknown what causes the elevated expression of HMGA1 in tumor cells (Cleynen et al, 2007).

HMGA1 has four transcriptional start sites with start site one and two playing the biggest role. Cleynen et al. demonstrated that *HMGA1* promoter contained three positive regulatory regions, one at transcription start site 1, one at transcription start site 2, and a distal region. The *HMGA1* promoter is very GC rich and lacks a TATA box. SP1 is a protein that plays a role in regulating the expression of genes without a TATA box. SP1 has four binding sites on the *HMGA1* promoter with the binding site at -62/-52 bp playing an essential role in basal promoter activity. Another protein that was shown to have a considerable effect on *HMGA1* promoter activity was AP1. AP1 has a binding site directly before the second transcription start site (Cleynen et al., 2007).

Cleynen et al. demonstrated that cells containing mutant Ras have detectable levels of HMGA1 while cells with wild type Ras do not. In preliminary results, inhibition of the Ras-Raf-MEK-ERK pathway leads to a downregulation of endogenous HMGA1. However, another group has proposed that HMGA1 regulates the Ras/ERK pathway (Cleynen et al., 2007).

While HMGA1 is overexpressed in many cancers, including all types of lung cancer (Lin & Peng, 2015), it has also been shown to promote senescence. Senescent cells can accumulate senescence-associated heterochromatic foci (SAHF) which prevents the activation of proliferative genes. SAHF have many proteins bound to them including HMGA1. Cells that expressed high levels of HMGA1 were shown to undergo acute cell cycle arrest and displayed features of cellular senescence including increased $p16^{INK4a}$ and p53, a senescent morphology, and elevated SA-β-galactosidase activity. These cells also acquired SAHF-like foci. However, when cells expressed short hairpin RNA against p16, they did not undergo HMGA1-induced arrest or form SAHF. These data suggest that high levels of HMGA1 are oncogenic if the senescence program is disabled (Narita et al., 2006).

2.0 Specific Aims

2.1 Introduction

Oncogene induced senescence (OIS) is anti-tumorigenic. Non-small cell lung cancer (NSCLC) cells need to bypass OIS in order to form tumors. Circulating angiotensinogen is produced in the liver and is converted to Ang I by the kidney-derived renin enzyme. Ang I is then converted to Ang II by the angiotensin-converting enzyme (ACE) on the surface of endothelial cells. Whether Ang II is locally produced in the lung in a renin/ACE-independent manner following oncogene activation and its role in OIS and transformation are unknown.

2.2 Preliminary Data

Oncogenic K-Ras (K-Ras^{G12V}) upregulates AGT mRNA and protein expression and increases Ang II levels in normal and NSCLC cells.

Hypothesis: Oncogenic K-Ras transcriptionally activates AGT in normal cells through KLF6 and in cancer cells in an HMGA1-dependent manner. This hypothesis will be tested through two aims.

Aim 1: Determine the role of KLF6 in K-Ras^{G12V}-dependent activation of the angiotensinogen promoter in normal cells.

To identify the region(s) of the AGT promoter that are activated by oncogenic K-Ras, three deletion mutants of the AGT promoter were made and cloned upstream of the luciferase

gene. NIH 3T3 cells were transfected with AGT deletion mutants or the wild type form of the angiotensinogen promoter followed by a luciferase assay to determine gene expression levels. *In silico* analysis showed three KLF6 binding sites on the AGT promoter. *KLF6* binding site deletion mutants of the AGT promoter were made and gene expression levels will be determined by a luciferase assay. To causally link KLF6 to oncogene-induced activation of the AGT promoter, K-Ras^{G12V}-induced activation of the AGT promoter will be measured following downregulation of KLF6 by siRNA.

Aim 2: Determine the significance of HMGA1 on the activity of the angiotensinogen promoter in oncogenic K-Ras expressing NSCLC cells.

Preliminary data show that AGT is over-expressed while KLF6 expression is dramatically downregulated in NSCLC cells. *In silico* analysis shows that the AGT promoter has two putative HMGA1 binding sites. To determine whether HMGA1 mediates AGT promoter activation in NSCLC cells, HMGA1 expression was quantified by immunoblotting analysis in A549 and H460 cells. To causally link HMGA1 function to AGT promoter activation, luciferase assays were performed in A549 cells expressing the AGT-luciferase construct following downregulation of HMGA1 by siRNA. Finally, to link STAT3 signaling, which is activated in lung cancer, to HMGA1-dependent transcription of AGT, levels of active STAT3 were measured in A549 and H460 NSCLC cells in which HMGA1 expression is downregulated by siRNA.

Significance: These investigations provide a novel and significant link between an oncogene (K-Ras) and the local production of biologically active molecules, i.e. Ang II, in the lung. This new information has the potential to impact the development of novel therapeutic interventions aimed at manipulating the anti- and pro-tumorigenic properties of oncogenic K-Ras

in cancer through local modulation of the angiotensin system in the lung. These interventions can be used to help eliminate a major public health problem, lung cancer.

3.0 Materials and Methods

3.1 Cell Culture

Normal NIH 3T3 and MEF cells were grown in DMEM containing 10% heat inactivated fetal bovine serum (FBS), 1% glutamine, and 1% antibiotics (penicillin and streptomycin). A549 and H460 cells were grown in F12 media containing 10% FBS (not heat inactivated), 1% glutamine, and 1% antibiotics (penicillin and streptomycin).

3.2 KLF6 Binding Site Deletion Mutants

KLF6 binding site deletion mutants were made using the primer sequences listed in Table 1. Figure 2 shows how the three deletion mutants were generated.

Table 1. KLF6 Deletion Primers for Angiotensinogen 1,000bp

Primer Name	Primer Sequence
1. h-ANG-5'-KpnI	GGCCGGGGTACCTCCTGGGTAATTTCATGTCTG
2. h-ANG-3'-HindIII	GGCCGGAAGCTTAGAACAACGGCAGTTCTTCC
3. h-ANG-Δ1-5'	GTCAGAAGTGGGTTGCCTAAGC
4. h-ANG-Δ1-3'	GCTTAGGCAACCCACTTCTGAC
5. h-ANG-Δ2-5'	GGGTACATCTCTGGGTCAGAAG
6. h-ANG-Δ2-3'	CTTCTGACCCAGAGATHTACCC
7. h-ANG-Δ3-3'HindIII	GGCCGGAAGCTTCCGGGTCACGATGCCCTATTTA

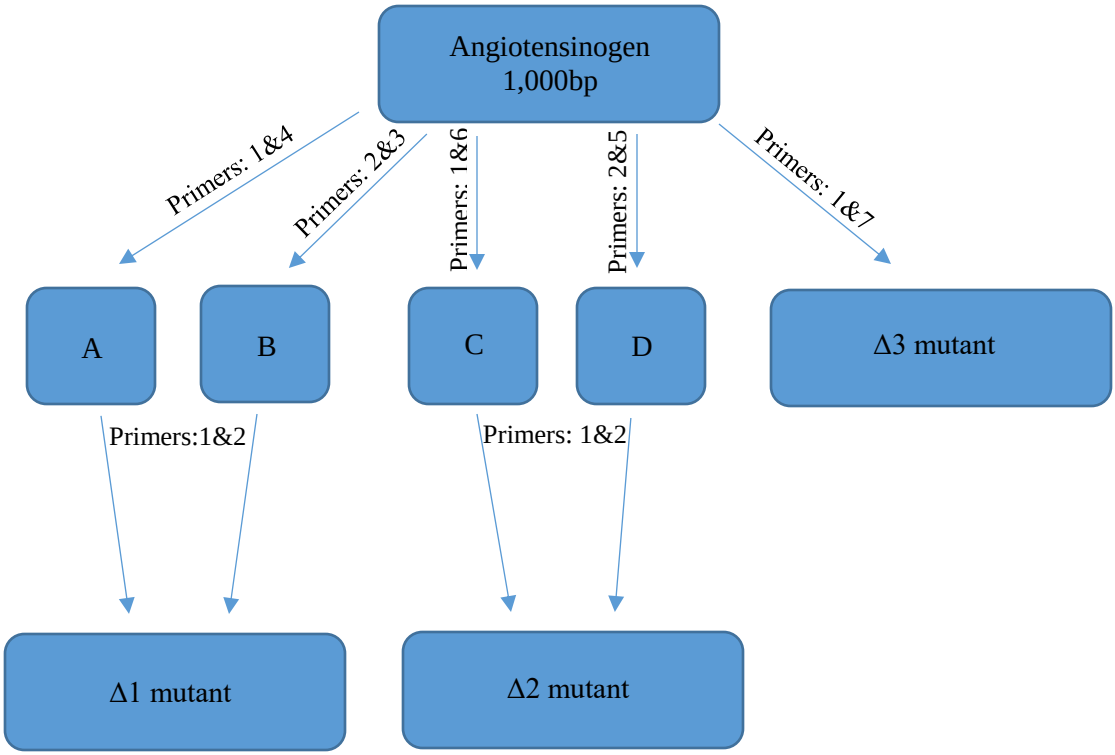

Figure 2. KLF6 Binding Site Deletion Mutants

Diagram showing how the three deletion mutants were constructed using angiotensinogen (-1,000bp/-1bp) as the template. Constructs were cloned into the p-TA-Luc vector.

PCR purification was performed on the three mutant products using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Overnight digestion at 37°C was performed using 24 µL of the purified PCR product, 3 µL NEBuffer 2.1, 1.5 µL HINDIII and H_2O up to 30 µL. 5 µg of angiotensinogen 1,000bp-pTA-Luc plasmid, 3 µL NEBuffer 2.1, 1.5 µL HINDIII and H_2O up to 30 µL was used to isolate the pTA vector. Gel extraction of digestion products was performed using QIAquick extraction kit (Qiagen, Valencia, CA) and 20 µL of H_2O to elute the DNA. An overnight digestion at 37°C was performed using 20 µL of the purified digested DNA, 3 µL CutSmart Buffer, 1.5 µL KpnI HF, and H_2O up to 30 µL. The single digestion samples were PCR purified using the same technique as mentioned above. Ligation was performed overnight at room temperature using 50 ng of vector (pTA-Luc) and either 30 ng of insert (1:3) or 10 ng of

insert (1:1) plus 1 μL buffer 10X, 0.5 μL T4 DNA ligase (New England Biolabs, Ipswich, MA), and H_2O up to 10 μL. DH5α cells were transformed by adding 50 μL of cells to the ligation tubes and leaving them on ice for 30 minutes. The cells were shocked at 42°C for 30 seconds and then put back on ice for two minutes. 300 μL of LB broth was added to the tubes, and they were put in the shaker for one hour at 37°C. The mixture was plated on agar plates containing ampicillin overnight at 37°C. Colonies were picked from the plates and added to 16 μL of STE 1X (200 μL NaCl, 100 μL Tris, and 20 μL EDTA in 10 mL of water). After also adding 16 μL phenolchloroform (bottom phase) to each tube, vortexing, and spinning at max speed for 8 minutes, the supernatant (10 μL) was taken out and combined with 2 μL of TAE 6X + RNase (1 mL TAE 6X + 20 μL RNase). The samples were run on a 0.8% agarose gel along with the undigested angiotensinogen 1,000bp-pTA-Luc plasmid to identify recombinants which were then selected and purified using the QIAGEN Plasmid Purification kit (Qiagen, Valencia, CA).

3.3 Luciferase Reporter Assay

3.3.1 KLF6 Deletion Mutant Transfection

NIH 3T3 cells were seeded in 6-well plates. At 80-90% confluency, the cells were transfected using 66 μl calcium phosphate with 1.8 μg of either angiotensinogen (cloned in pTA-Luc Vector), Δ1 mutant, Δ2 mutant, or Δ3 mutant and 1 μg of β-gal. 1.8 μg of either the empty pLVX vector or pLVX-K-Ras^{G12V} were then added to the tube. 66 μL of HeBS was added to the tubes and allowed to incubate at room temperature for 20 minutes. Following the incubation, 132

μL was added to each well of cells. Cells were incubated at 37°C for 48 hours after transfection before the luciferase assay was performed.

On the day of the luciferase assay, the cells were washed twice with cold 1X PBS. They were put on a shaker in a cold room for 30 minutes with 500 μL of extraction buffer (10 mL glyceryl monooleyl ether (GME), 10 μL DTT, and 100 μL triton) in each well. After 30 minutes, the cells were scraped and spun down for five seconds at maximum speed. 200 μL of sample was added to 300 μL of the ATP mix (9 mL GME, 1.8 mL potassium phosphate, 12 μL DTT, and 100 μL ATP), and 100 μL of sample was added to 600 μL of Z-buffer (10 mL Z-buffer and 35 μL β-mercapthoethanol) in duplicate. The samples in the ATP mix were used for the luciferase assay using a luc solution (2 mL luciferin, 8 mL GME, and 100 μL DTT). 300 μL of chlorophenol red-β-galactopyranoside (CPRG) (0.016 g CPRG in 4 mL distilled H_2O) was also added to the samples in the Z-buffer and were incubated at 37°C for 30 minutes. After 30 minutes, the sampled were read at 574nm using an Ultraspec 2100 pro for β-gal normalization.

3.3.2 KLF6 siRNA Transfection

NIH 3T3 cells were seeded in 35mm dishes. At 60-80% confluency, the cells were transfected with 40 pmol of either scrambled siRNA or KLF6 siRNA using the Lipofectomine RNAiMAX Reagent Protocol 2013 (Thermofisher, Waltham, MA). After 24 hours, each plate was split into 6-well plates and incubated for an additional 24 hours. The following day the cells were transfected again with a mammalian expression vector containing β-gal to act as a control vector, angiotensinogen 1,000bp pTA-Luc, and either the empty pLVX vector or pLVX-K-Ras^{G12V} vector according to the calcium phosphate protocol stated above. Forty-eight hours after the second transfection, the luciferase assay was performed. KLF6 knockdown was verified by western blot.

3.3.3 HMGA1 siRNA Transfection

A549 cells were seeded in 6-well plates. At 65% confluency, they were transfected using the Lipofectamine RNAiMAX Reagent Protocol (Thermofisher, Waltham, MA). 40 pmol of siRNA was used. After 48 hours when the cells were 80% confluent, they were transfected with 1 μg of β-gal and 2 μg of angiotensinogen 1,000bp pTA-Luc using the Lipofectamine 3000 Reagent Protocol (Thermofisher, Waltham, MA). 7 μL of lipofectamine 3000 reagent, 6 μL P3000, and 3 μg of DNA were used. Seventy-two hours after the second transfection, the luciferase assay was performed. HMGA1 knockdown was verified by western blot.

3.4 Western Blot

A549 and H460 cells were seeded in 6-well plates and *HMGA1* expression was knocked down using the Lipofectamine RNAiMAX Reagent Protocol as described above. Cells were collected 24, 48, and 72 hours after treatment. Cells were collected in boiling sample buffer (1X LB). The samples were run on a 12.5% acrylamide gel and transferred to BA83 nitrocellulose membranes (Schleicher & Schüll). The blots were incubated in a TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.2% Tween 20) solution containing 2% powdered skim milk and 1% BSA for 1 hour and 15 minutes. The blots were rinsed three times with TBST and incubated for 2 hours at room temperature and then placed in the cold room overnight with the primary antibody monoclonal total STAT3 (Cell Signaling, catalogue #4904, diluted), β-actin (Santa Cruz Biotechnology, catalogue #47778, diluted 2000-fold), polyclonal PhospoSTAT3 (Cell Signaling, catalogue #9131, diluted 1000-fold), AGT (Cell Signaling, catalogue #79299, diluted

1000-fold), or monoclonal HMGA1 (Cell Signaling, catalogue #7777, diluted 1000-fold). The following day, the blot was washed three times with TBST and incubated for 1 hour and 15 minutes with either goat anti-rabbit IgG (BD Pharmingen, catalogue #554021, diluted 2500-fold) or goat anti-mouse IgG (Invitrogen, catalogue #A32723, diluted 5000-fold). Bound antibodies were detected using an ECL detection kit (Thermofisher, Waltham, MA).

4.0 Results

4.1 K-Ras^{G12V} activates the AGT promoter through KLF6

Our lab has previously shown that oncogenic K-Ras upregulates AGT mRNA expression. We wondered whether K-Ras^{G12V} could transcriptionally activate the AGT gene promoter. To do this, we took a luciferase construct and cloned the first 1,000bp of the human AGT promoter upstream of the luciferase gene [AGT(1,000)-LUC]. Figure 3A shows that K-Ras^{G12V} activated the AGT promoter in cells transfected with AGT(1,000)-LUC. AGT deletion mutants were generated to determine which portion of the AGT promoter played a significant role in its activation by oncogenic K-Ras. Figure 3C shows the relative activation of the AGT promoter when cells were transfected with one of the AGT deletion mutants (Figure 3B) and either pLVX or K-Ras^{G12V}. The data shows that there is a significant decrease in activation of the AGT promoter in the -500bp region upstream of the start codon in response to K-Ras^{G12V}. In silico analysis showed multiple transcription factor binding sites including three putative binding sites KLF6. The binding sites for KLF6 were located at positions -445bp, -354bp, and -15bp from the start codon (Figure 3D).

To test the hypothesis that oncogenic K-Ras transcriptionally activates AGT in normal cells though KLF6, we generated mutant forms of the human AGT promoter in which each KLF6 binding site was deleted (Figure 3D). NIH 3T3 cells were transfected with these KLF6 deletion mutants and either K-Ras^{G12V} or pLVX. Figure 3E shows that cells co-transfected with K-Ras^{G12V} and the Δ1 mutant had a significant decrease in AGT activation compared to the control.

In support of the results seen using the luciferase reporter assay for the deletion mutants, we found that K-Ras^{G12V} upregulated KLF6 mRNA (Figure 4A) and protein expression (Figure 4B) in fibroblasts (WI-38 and MEFs). We also demonstrated that downregulation of KLF6 by siRNA prevented oncogenic K-Ras-induced activation of the AGT promoter (Figure 4C) and upregulation of AGT protein expression (Figure 4D).

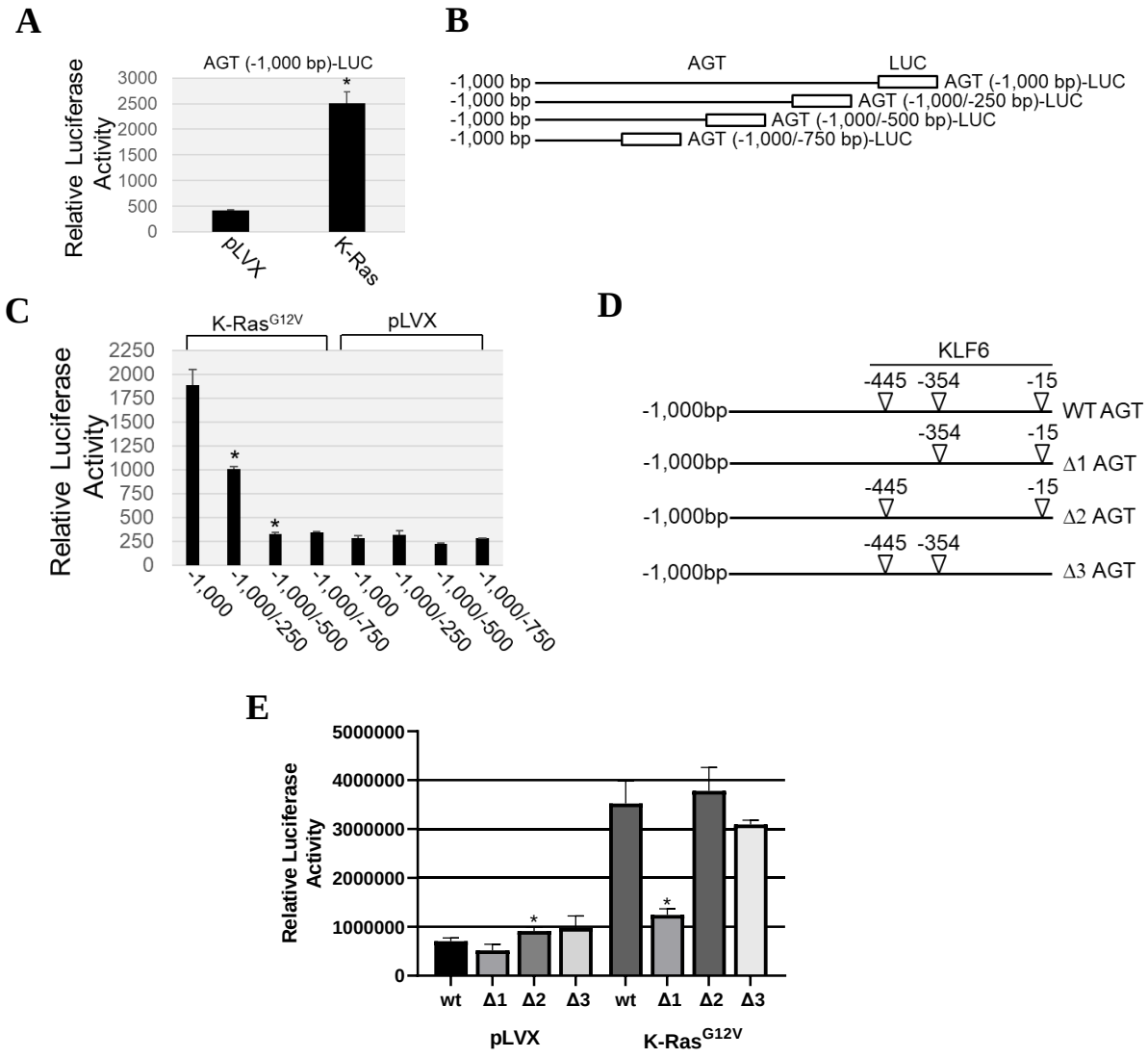

Figure 3. Activation of AGT Promoter by K-Ras^{G12V}

The human AGT promoter is activated by K-Ras^{G12V}. (A) Relative luciferase activity of normal fibroblast was determined by transfecting cells with AGT (1,000)-LUC and either pLVX or K-Ras^{G12V}. Data show increased activation of AGT when K-Ras^{G12V} is present. (B) AGT mutant constructs that were generated using PCR and cloned into a pTA-Luc vector. (C) Relative luciferase activity of normal fibroblasts was determined by transfecting cells with the AGT mutant constructs shown in (B) and either pLVX of K-Ras^{G12V}. Data shows that -1000/-250 and -1000/-500 have a significant reduction in AGT activation. (D) AGT promoter constructs missing individual KLF6 binding sites that were generated using PCR and cloned into a pTA-Luc vector. (E) Relative luciferase activity of normal fibroblasts determined by transfecting cells with the AGT constructs in (D) and either pLVX or K-Ras^{G12V}. Data shows that the Δ1 construct causes a significant reduction in AGT activation. Values in (A), (C) and (E) represent means ± SEM; statistical comparisons were made using the student's t-test. *P<0.001.

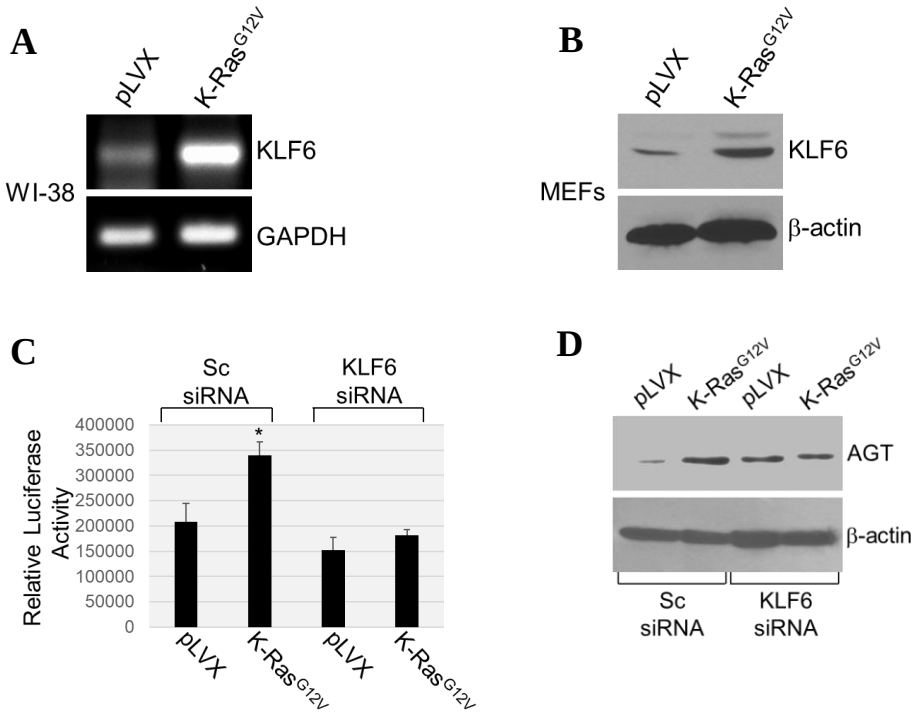

Figure 4. Downregulation of KLF6 Prevents AGT Activation

AGT is activated by oncogenic K-Ras via KLF6. (A) RT-PCR of WI-38 cells transfected with K-Ras^{G12V} show an increase in KLF6 mRNA compared to cells transfected with pLVX. (B) Immunoblotting of MEFs transfected with K-Ras^{G12V} show an increase in KLF6 protein levels. (C) Cells transfected with KLF6 siRNA and K-Ras^{G12V} did not show a significant increase in AGT activation. (D) Immunoblotting of cells from (C) show lower AGT protein levels when transfected with KLF6 siRNA compared to scrambled siRNA. Values in (C) represent means ± SEM; statistical comparisons were made using the student's t-test. *P<0.001.

4.2 HMGA1 upregulates AGT expression in NSCLC cells

Previous data generated from the lab has shown that K-Ras^{G12V} promotes premature senescence through the upregulation of the AGT/Ang II pathway. Since cellular senescence is a tumor suppressor mechanism, we looked to see whether activation of this pathway was inhibited in lung cancer cells that carried oncogenic K-Ras. To our surprise, what we discovered was that AGT expression was elevated in A549 and H460 lung cancer cells endogenously expressing oncogenic K-Ras compared to normal human bronchial epithelial (NHBE) cells (Figure 5A). We looked at the expression of KLF6 in these cell types and found that KLF6 was downregulated (Figure 5A), suggesting that KLF6 was not the transcription factor mediating AGT expression in NSCLC cells. *In silico* analysis showed two putative binding sites for the transcription factor HMGA1 at positions -996 and-651 (Figure 5B). We show in Figure 4A that HMGA1 expression was elevated in both A549 and H460 cells. To test the hypothesis that HMGA1 upregulates AGT expression in NSCLC cells, we knocked down expression of HMGA1 using siRNA in A549 cells. Following knockdown of HMGA1 expression, we transfected the cells with the full length (-1,000bp) AGT promoter/luciferase construct and performed a luciferase reporter assay to quantify activation of the AGT promoter (Figure 5C). We show that cells treated with siRNA against HMGA1 had a significant decrease in AGT promoter activity, as compared to cells treated with scrambled siRNA.

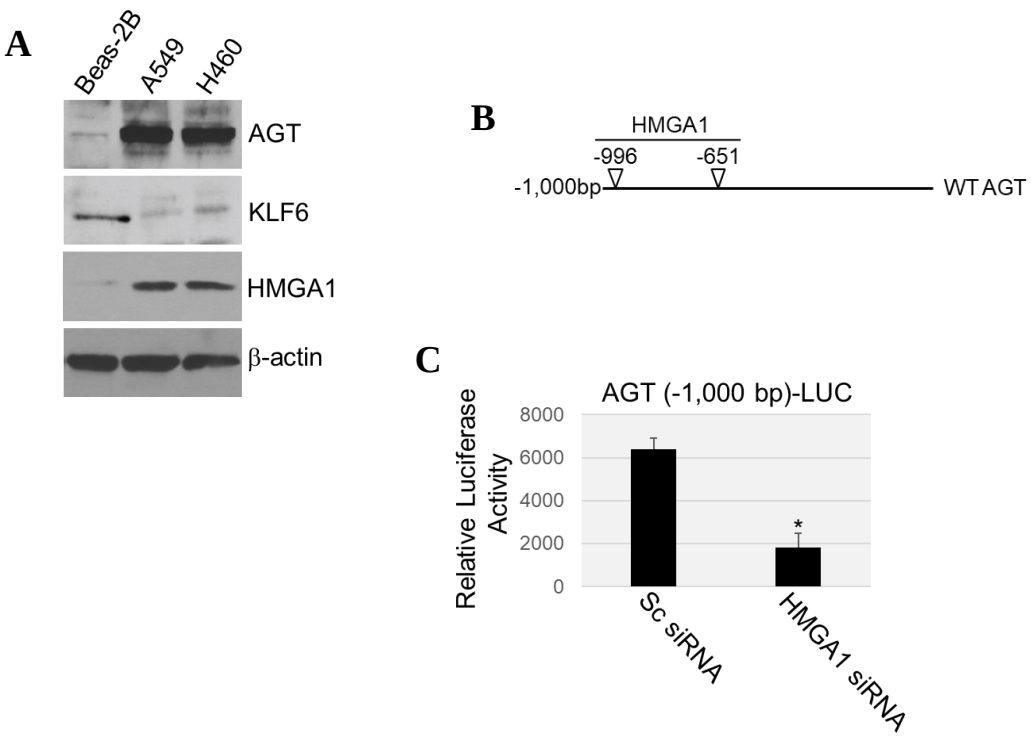

Figure 5. HMGA1 is Upregulated in NSCLC Cells and Activates AGT Promoter

AGT is activated by oncogenic K-Ras via HMGA1 in NSCLC cells. (A) Immunoblotting comparing protein levels in normal cells vs NSCLC cells. NSCLC cells A549 and H460 show an increase in AGT and HMGA1 protein levels while also showing a decrease in KLF6 protein levels compared to normal cells Beas-2B. (B) AGT promoter showing the two putative binding sites of HMGA1. (C) A549 cells transfected with HMGA1 siRNA show a significant decrease in AGT promoter activation. Values in (C) represent means ± SEM; statistical comparisons were made using the student's t-test. *P<0.001.

4.3 HMGA1 activates STAT3 in NSCLC cells

Phosphorylation on tyrosine 705 (Y705) activates STAT3. This activation causes STAT3 to translocate into the nucleus and bind to DNA causing transcription of a number of different genes (Levy & Lee, 2002). Since NSCLC is characterized by elevated STAT3 expression or activity (Johnston & Grandis, 2011) and have elevated AGT expression, we hypothesized that activation of STAT3 is downstream of the K-Ras^{G12V}-initiated and AGT/Ang II-mediated activation of the AT_1 receptor. Figure 6A shows increased P(Y705)-STAT3 in A549 cells compared to Beas-2B cells. Since HMGA1 protein levels are elevated in A549 cells and cause an increase in AGT activation, we wanted to test whether HMGA1 activates STAT3 in NSCLC cells. We treated A549 and H460 cells with either scrambled siRNA or siRNA against HMGA1. Immunoblotting (Figure 6B, C) shows that P-STAT3 protein levels are lower in both cell types when treated with siRNA against HMGA1 compared to cells treated with scrambled siRNA.

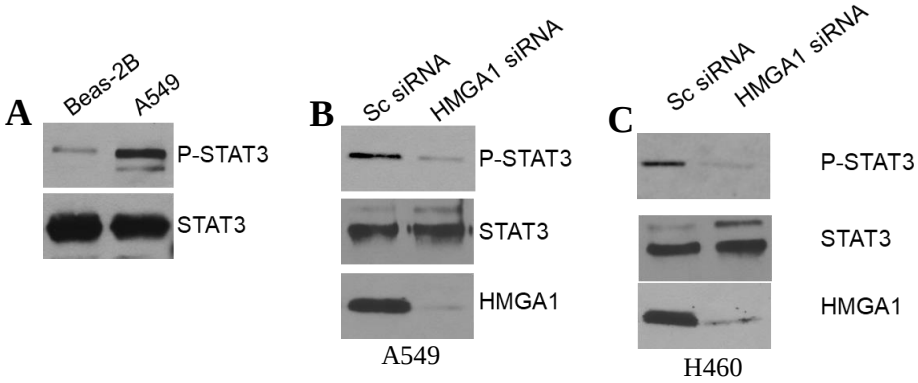

Figure 6. HMGA1 activates STAT3 in A549 and H460 NSCLC cells

Activated STAT3 levels are influenced by HMGA1 protein levels. (A) Immunoblotting of NSCLC cells shows an increase in P-STAT3 levels compared to normal cells. (B) Immunoblotting of A549 cells transfected with HMGA1 siRNA shows a reduction of P-STAT3 levels compared to scrambled siRNA-transfected cells. (C) Immunoblotting of H460 cells transfected with HMGA1 siRNA shows a reduction of P-STAT3 levels compared to cells transfected with scrambled siRNA.

5.0 Discussion

The goal of this project was to determine whether KLF6 and HMGA1 play a role in activating AGT in normal fibroblasts over expressing oncogenic K-Ras (K-Ras^{G12V}) and lung cancer cells that already express the oncogenic form of K-Ras^{G12V}. Since Ang II has been implicated in inducing SIPS by its over production of ROS (Herbert et al., 2008), determining the major players of AGT promoter activation may lead to new ways in which we treat cancer.

WI-38 cells and MEFs over-expressing K-Ras^{G12V} were shown by our laboratory to have upregulation of AGT mRNA and protein expression compared to control cells which expressed the empty pLVX vector. These data provided the rationale to study transcription factors that have binding sites on the AGT promoter. We find that the region most responsible for this K-Ras induced activation of AGT is in the -1 to -500bp region from the start codon. Analysis of this area shows three putative binding sites for the transcription factor KLF6. By deleting each KLF6 binding site of the AGT promoter individually and cloning it into a pTA-Luc vector, we found which binding site plays the biggest role in activating the AGT promoter. The binding site that causes the greatest decrease in AGT promoter activation is located at -445bp. Consistent with these findings, we also show that downregulating KLF6 using siRNA inhibits K-Ras-induced activation of the AGT promoter. siRNA against KLF6 does not abolish activation of the AGT promoter; this can be explained by the fact that i)- KLF6 siRNA does not eliminate KLF6 expression and therefore residual levels of KLF6 remain in these cells; and ii)- the AGT promoter has additional binding sites for other transcription factors which could mediate AGT promoter activation in a K-Ras^{G12V}-dependent manner.

Oncogenic K-Ras induces senescence in normal cells and preliminary findings from our laboratory show that K-Ras^{G12V} promotes senescence in fibroblasts in an Ang II-dependent manner. Thus, the next question we investigated was if the AGT/Ang II signaling pathway was inhibited in lung cancer cells carrying oncogenic K-Ras, which have bypassed cellular senescence. We thought we would see lower levels of AGT mRNA and protein which would also lead to lower levels of Ang II. In contrast to our hypothesis, we found that AGT expression was actually elevated in NSCLC cells, as compared to NHBE cells. We also discovered that elevation of AGT expression is not mediated by KLF6 in these cells as KLF6 expression is extremely downregulated in both A549 and H460 cells. However, another transcription factor, HMGA1, is highly expressed in NSCLC cells and the AGT promoter has two putative HMGA1 binding. By using siRNA, we downregulated HMGA1 expression in NSCLC cells and showed that this downregulation caused a decrease in AGT promoter activation. HMGA1 is a transcription factor that is expressed at very high levels in embryogenesis and is almost undetectable in adults. In order for NSCLC cells to overcome OIS, they likely need to accumulate mutations in both tumor suppressor genes, such as KLF6, and proto-oncogenes. We speculate that a mutation of the HMGA1 gene could upregulate HMGA1 protein expression in NSCLC cells allowing them to bypass OIS, possibly through the ability of HMGA1 to regulate growth pathways such as the AGT/Ang II signaling.

We also show that STAT3 is activated when HMGA1 is highly expressed in cells. STAT3 is a transcription factor that becomes active when the tyrosine at position 705 is phosphorylated. P-STAT3 is then able to translocate to the nucleus where it plays a role in regulating transcription of a host of different genes. STAT3 has also been implicated in cancer. Constitutively active STAT3 has been shown to lead to fibroblast transformation, which suggests

it behaves like an oncogene (Levy & Lee, 2002). Given the key role that STAT3 plays in tumorigenesis, we looked at the functional relationship between HMGA1 and STAT3 activation. First, we show that STAT3 is elevated in NSCLC cells compared to Beas-2B cells as is HMGA1. To test whether STAT3 signaling is downstream of the AGT/Ang II-mediated activation of AT_1 receptor, we transfected both A549 and H460 cells with HMGA1 siRNA. We show that when both cell types are transfected with HMGA1 siRNA there is a decrease in P-STAT3 compared to cells transfected with scrambled siRNA. To show that activation of STAT3 is at least partly dependent upon activation of the AT_1 receptor, we treated cells with losartan, which is a competitive antagonist for the AT_1 receptor. Cells treated with losartan showed inhibition of P-STAT3 (not shown). Consistent with this data, over-expression of the AT_1 receptor activated STAT3 (not shown).

We not only found that the AGT promoter is activated in both normal and NSCLC cells by oncogenic K-Ras but levels of its downstream product, Ang II, are also elevated. AGT is part of the classical renin-angiotensin system which regulates blood pressure. Two enzymes, renin and ACE, are involved in converting AGT to Ang I and Ang I to Ang II, respectively. Interestingly, out data show that both normal lung and NSCLC cells do no express either renin or ACE. We found that up-regulation of Ang II levels, following the K-Ras^{G12V}-induced activation of the AGT promoter is mediated by cathepsin D, chymase, and tissue plasminogen activator (TPA) (not shown), which can mediate the conversion of AGT to Ang I, Ang I to Ang II, and AGT directly to Ang II, respectively. Thus, we identified a novel cell autonomous and renin/ACE-independent pathway that promotes the oncogenic K-Ras-induced synthesis of Ang II in both normal and cancer cells (Figure 7).

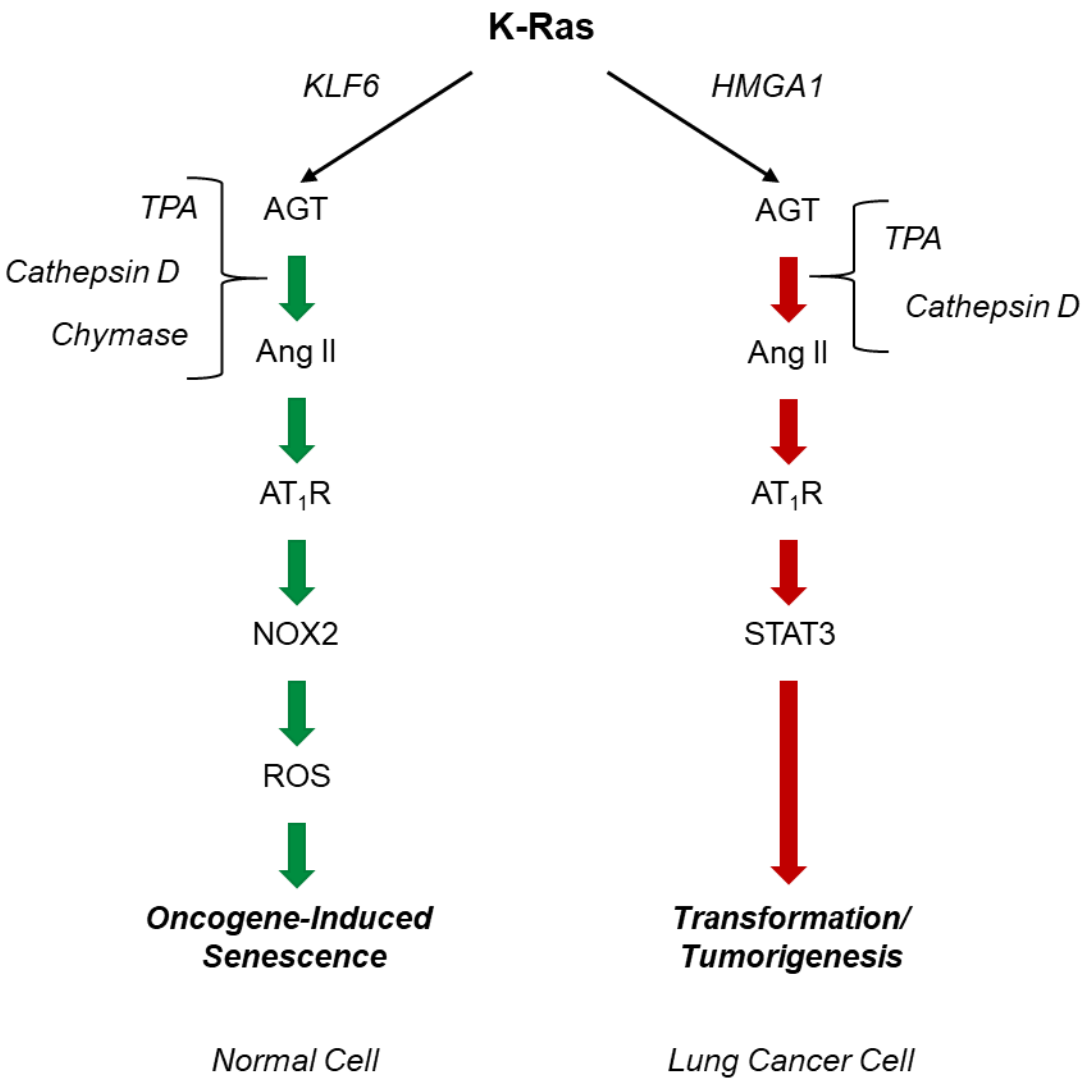

Figure 7. Oncogenic K-Ras Induced Synthesis of Ang II Independent of Renin and ACE

Left arm of the pathway shows K-Ras induced AGT promoter activation in normal cells which causes OIS via production of ROS. The right arm of the pathway shows K-Ras induced AGT promoter activation in lung cancer cells which causes tumorigenesis via activation of STAT3.

In this study, we show that both KLF6 in normal cells and HMGA1 in NSCLC cells are transcriptional regulators of the angiotensinogen promoter following oncogenic K-Ras stimulation. On one hand, our data provide evidence that oncogenic K-Ras induces senescence in non-transformed cells through the activation of the AGT promoter in a KLF6-dependent fashion. Thus, our findings indicate that the AGT/Ang II signaling pathway plays a role not only in maintaining blood pressure but also in cancer prevention by inducing OIS in normal cells. On the other hand, our data indicate that oncogenic K-Ras promotes transformation of NSCLC cells through HMGA1-dependent and AGT/Ang II/AT_1-R/STAT3-mediated signaling. This study provides novel mechanistic insights into the pleiotropic role of oncogenic K-Ras in normal and NSCLC cells and identifies novel targets of therapeutic potential for cancer treatment.

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