STUDY OF THE ROLE OF THE ANGIOTENSIN II (ANG II) TYPE 2 RECEPTOR (AT_2) IN LUNG TUMORIGENESIS

by

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Abstract

Lung cancer mortality is the highest among all cancer–associated deaths. Despite early detection and treatment, prognosis of this disease remains poor. Therefore, development of new therapeutic agents and effective treatment procedures are urgently needed. Endogenous Angiotensin II (Ang II) type 2 receptor (AT_2), one of two isoforms of Ang II, has been shown to mediate apoptosis. Nanoparticle delivery systems make possible targeted drug delivery and controlled release of therapeutic molecules and genes. Thus, the aim of this study was to determine the anti-cancer effect of the over-expressed AT_2 gene on lung adenocarcinoma cells *in vitro* using adenoviral vector (Ad-) and nanoparticle (NP-) based gene delivery systems. This study showed that over-expression of Ad-AT_2 induced cancer cell-specific apoptosis in several human lung adenocarcinoma cell lines with minimal effect on normal lung epithelial cells. Ad-AT_2 significantly attenuated multiple human lung cancers' cell growth (A549 and H358) *in vitro* compared to the control viral vector, Ad-β-galactosidase (Ad-LacZ) when examined by direct cell count. The growth attenuation effect was detected as early as 24 hours after Ad-AT_2 transfection and lasted 12 days. Western Blot analysis revealed the activation of the caspase pathway. Examination for Annexin V by flow cytometry also confirmed activation of the apoptotic pathway via AT_2 over-expression. Similarly, AT_2 cDNA encapsulated poly(DL-lactide-*co*-glycolide) (PLGA) biodegradable nanoparticles (NPs) were shown to be effectively taken up into lung cancer cells. Surface conjugation of the angiotensin II peptide significantly stimulated uptake of the particles. This PLGA vector-dependent AT_2 transfection was effective in sustained gene expression and resultant cell death. These results indicate that the AT_2 over-expression effectively attenuated growth of lung adenocarcinoma cells through activation of intrinsic apoptosis. Since PLGA safety has been proven, whereas adenoviral vectors have several drawbacks in safety, the Ang II conjugated PLGA nanoparticles may be a better therapeutic gene delivery system. Therefore, it is concluded that the discovery of AT_2 DNA encapsulated PLGA conjugated with the Ang II peptide is a potentially useful tool for lung cancer gene therapy.

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Acknowledgements

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Dedication

I would like to dedicate this thesis to my fiancé, Josh, and to my family for all of their love and support as I have pursued my Master's degree.

Chapter 1 - Introduction

Lung cancer remains the leading cause of tumor-related mortality despite ongoing efforts to find effective treatments. Data from the American Cancer Society indicate that while the overall incidence of lung cancer is declining, it continues to rise in women. In 2007, the American Cancer Society estimated that 213,380 persons in the U.S. will develop new cases of lung cancer and that 160,390 persons will die from it.

Studies show that tobacco smoke is a likely cause of the majority of lung cancer cases (1). Cigarette smoke contains multiple carcinogenic agents, not the least of which are tobacco-specific nitrosamines, the most carcinogenic agents in rodents (2). While environmental factors are important, genetic factors also play an important role in this disease. Gene mutations that occur in tumor suppressor genes such as p53, p15/16, MYO18B, cyclin kinase inhibitory genes and mismatch repair genes have been extensively explored and implicated in the origin of lung cancer (3). Nevertheless, the overall incidence of lung cancer is sporadic even within the smoking population (less than 20% of cigarette smokers develop lung cancer). Therefore, the presence of other mechanisms that trigger lung cancer is evident. Further clarification of such factors is essential.

Angiotensin II (Ang II) is the key effector of the renin-angiotensin system, which maintains blood pressure, body fluid and electrolyte homeostasis (4). The Ang II receptor has two major isoforms, and their signaling is associated with cell proliferation and apoptosis (5). The first, AT_1, is expressed in a wide variety of tissues, and its signaling functions in a variety of pathophysiological reactions, including constriction of blood vessels, induction of cell proliferation and expression of proto-oncogenes such as c-fos, c-myc and c-jun (6). The second, AT_2, is abundantly expressed in fetal tissues, but its expression declines rapidly after birth. Previous studies have shown that AT_2 receptor signaling counteracts the AT_1 signaling-mediated biological effects, including inhibition of cell proliferation and induction of apoptosis (7). Their delicate balance, and therefore the balance between proliferation and apoptosis, plays an essential role in the pathophysiology of various diseases (5).

Several studies have demonstrated that AT_2 receptor signaling has anti-proliferative effects through apoptosis induction in a variety of cell lines, such as vascular smooth muscle

cells, cardiomyocytes, neuronal cells, fibroblasts and endothelial cells (8-11). In an *in vivo* model, the pro-apoptotic effect of the AT_2 receptor on vascular smooth muscle cells was demonstrated (10). A recent study has indicated that an interferon regulatory factor (IRF) exists in the AT_2 receptor promoter region (12). IRF-1 has been shown to act as a tumor suppressor gene in human breast cancer cells, implying that the AT_2 receptor may also have tumor suppressor properties. Furthermore, it is reported that the expression of the AT_2 receptor itself signals for apoptosis without requiring the ligand Ang II (8). These findings led to the hypotheses that the induction of the AT_2 receptor into lung cancer cells or tumor tissue induces apoptosis and that the AT_2 receptor is a potential therapeutic gene to control lung cancer.

Generally, gene therapy-induced tumor growth inhibition requires sustained and robust expression of the transgene in order to provide efficient therapy (13). This study explored two different forms of gene delivery: (1) a replication deficient adenovirus and (2) a poly(lactide-*co*-glycolide) (PLGA) biodegradable nanoparticle. Use of adenoviral vectors is a potentially effective strategy as they allow strong expression of transgenes, such as angiotensinogen and inhibitors-of-apoptosis-proteins (IAPs), in a variety of tissues, including tumor tissue (14, 15). Viral vectors are currently employed in more than 70% of clinical gene therapy trials worldwide and show promising results in preclinical cancer treatment (16, 17). However, their clinical applications are limited by their efficacy in that a viral vector is rapidly cleared from circulation (17). Viral vectors are also immunogenic, as many cases of viral infection have been reported (18). Nanoparticles present another viable option as a gene delivery vehicle. They can be designed to slip between intercellular spaces, enter cells, or transport directly through biological barriers to access disease sites. Additional research shows that nanoparticles protect the encapsulated gene from potential degradation (19). Recently, Son and Kim (2007) have demonstrated the therapeutic efficacy of Interleukin-12 encapsulated nanoparticles in colon adenocarcinoma tumor bearing mice.

The present study examined the effect of human AT_2 receptor overexpression on non-small cell lung cancer (NSCLC) cells using a replication deficient adenovirus and biodegradable nanoparticles as gene delivery vehicles. Chapters 2, 3 and 4 provide background information essential to a thorough understanding of the research presented. Namely, they discuss angiotensin II and its receptors, non-small cell lung cancer and gene therapy, respectively. Chapter 5 highlights the research regarding the adenoviral vector. Chapter 6 details the PLGA

nanoparticle findings. Chapter 7 provides a brief overview of future goals and intentions for this study. Finally, Chapter 8 summarizes the conclusions drawn from the study and provides an in-depth discussion of AT_2 receptor overexpression effects on lung cancer cells and its future application to cancer patients.

Chapter 2 - Angiotensin II and Its Receptors

The renin-angiotensin system plays a key role in fluid homeostasis and in blood pressure control (20). Circulating renin, an enzyme produced by the kidneys, cleaves circulating angiotensinogen to create angiotensin I. Angiotensin II (Ang II) is then produced by the Angiotensin I-converting enzyme (ACE). It has been shown that many tissues, including lung tissue, possess the capacity to generate Ang II locally, and this locally produced Ang II plays an important role in a variety of cell functions (21). Ang II is responsible for a plethora of biological actions, which include but are not limited to renal sodium retention, vasoconstriction, aldosterone release and cell proliferation (22). Ang II has two distinct receptors, type 1 (AT_1) and type 2 (AT_2), classified as G-protein-coupled seven transmembrane receptors (23).

The major isoform, AT_1, is found predominantly in a wide variety of adult tissues. AT_1 is primarly responsible for most Ang II-dependent actions in cardiovascular and renal tissues. The second isoform, AT_2, has limited expression in adult tissues but has very high expression in fetal tissues (24).

The relationship between AT_1 receptor signaling and cancer has recently been investigated. AT_1 receptor overexpression has been documented in a variety of cancers including breast, pancreatic, ovarian, melanoma, bladder and prostate cancer (24, 25). The AT_1 receptor stimulates growth factor pathways such as tyrosine kinase phosphorylation and induces phospholipase C, which leads to downstream activation of proteins such as mitogen-activated protein kinase (MAPK), jun N-terminal kinase (JNK) and signal transducers and activators of transcription protein (STAT), which upregulate cell growth (24). AT_1 receptor signaling also upregulates the vascular endothelial growth factor (VEGF) not only in vascular endothelial cells but also in cancer stromal cells such as fibroblasts and infiltrated macrophages found in the tumor microenvironment. Ang II mediated AT_1 receptor signaling also transactivates the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor (PDGFR) and the insulin-like growth factor receptor (IGFR) (26). EGFR is overexpressed in several epithelial malignancies; overexpression has been detected in up to 90% of all tumors (27). PDGFR is also found to be overexpressed in many carcinomas; PDGFR expression was in 95 –

100% of all head and neck tumor samples (28). In breast cancer, IGFR expression was nearly 10-fold higher than in normal breast tissue (29). Thus, the AT_1 receptor promotes cell growth and tumor growth.

The relationship between the AT_2 receptor and cancer, however, has yet to be clarified. In adult tissues, AT_2 receptor expression has been shown to be upregulated in wound healing and tissue remodeling (30). Previous studies also indicate that AT_2 receptor signaling counteracts the functions of the AT_1 receptor (7). The AT_2 receptor has been shown to inhibit cell proliferation and stimulate apoptosis in cardiovascular and neuronal tissues *in vitro* (23). Furthermore, the AT_2 receptor possesses the unique characteristic in that it does not require its ligand, Ang II, for signaling activation (8, 31). Recent research indicates that tumors in AT_2-null mice grow faster than those in wild-type mice (32). Therefore, the AT_2 receptor may contribute to tumor growth inhibition. Additional research recently showed strong expression of the AT_2 and AT_{1a} receptors in adenomatous hyperplastic epithelium (33). AT_2 receptor expression was also significant in vascular endothelial cells in small pulmonary vessels adjacent to the adenomatous lesion (33). This suggests that the angiotesin II receptors' expression potentially plays an important role in lung cancer. Therefore, the AT_2 receptor is a potential target for lung cancer therapy.

Chapter 3 - Non-Small Cell Lung Cancer

Lung cancer is the leading cause of mortality in both men and women (34). Lung cancer can be categorized into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is generally aggressive but accounts for less than one-quarter of all lung cancer patients. NSCLC, on the other hand, accounts for 75 - 80% of lung cancers (35). NSCLC generally grows slowly and may be curable at early stages; however, it shows limited response to chemotherapeutic treatment. It can be further categorized into three separate subtypes: *squamous cell carcinoma* (also called *epidermoid carcinoma*), *adenocarcinoma* and *large cell carcinoma* (36).

The first, squamous cell carcinoma, derives from the cells that replace injured or damaged cells in the epithelium of the bronchi, the major airways of the lung. Currently, it contributes to approximately 25 – 40% of all lung cancers (37). Squamous carcinomas are known to overexpress IAPs which are associated with resistance to chemotherapeutic agents such as carboplatin, cisplatin (cDDP), etoposide and cytosine arabinoside (38). They also frequently express p53 gene mutations (35).

The second, adenocarcinoma, accounts for 30 - 50% of all NSCLC lung cancers and is the most common type in developed countries. Adenocarcinomas originate from bronchial epithelia and/or type II alveolar epithelial cells. This cancer is virtually undetectable until the aggressive late stages, after which adenocarcinomas are typically fatal (39).

The third, large cell carcinoma, consists of only 10 - 20% of all lung cancers. According to the National Cancer Institute (NCI), large cell carcinoma is defined as "lung cancer in which the cells are large and look abnormal when viewed under a microscope." NCI also indicates that any cancer that cannot be classified as either squamous or adenocarcinoma is termed large cell carcinoma. This carcinoma tends to grow rapidly and metastasize early (40).

Currently, there are five standard treatments for lung cancer: (1) surgery, (2) radiation therapy, (3) chemotherapy, (4) laser therapy and (5) photodynamic therapy (40). However, despite aggressive research in the development of these therapies, the overall survival rate for

patients with lung cancer is less than 15% (Fig. 3.1) (34). Clearly new therapeutic options are urgently needed.

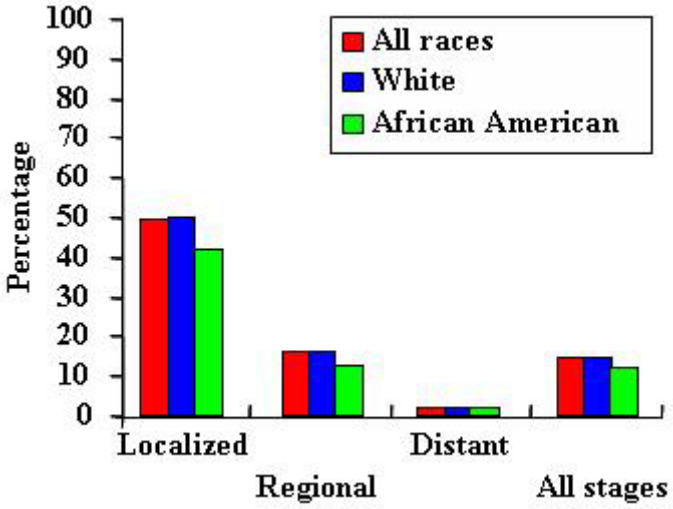

Figure 3.1 Five year survival rate for lung and bronchus cancers.

(Recreated from *Cancer Statistics, 2007*, CA Cancer J Clin.)

Chapter 4 - Gene Therapy

Gene therapy is an alternative to surgical removal of cancerous tissue, chemotherapy and radiation therapy. Gene therapy is, by definition, "the genetic modification of cells of a patient in order to fight a disease" (41). Gene therapy can either be the introduction of new genetic material or the manipulation of existing genetic material. In both cases, the three essential goals of gene therapy for cancer are as follows:

(1) to suppress cancer growth,

(2) to inhibit metastasis and

(3) to direct the therapeutic effects toward cancer cells only.

However, new genes cannot be directly delivered to cells: a carrier, or vector, is needed. This is due to the fact that the half-life of naked DNA in the blood is at most minutes (18). Commonly used gene vectors include the *retrovirus* and the *adenovirus*. Viral vectors are, in a sense, the ideal delivery system as they already possess all tools essential for cell binding, internalization and uptake (18).

The retrovirus is advantageous in that it is well understood and therefore, easily managed. It can include up to 9 kilobytes of new genetic material. Retrovirus studies have demonstrated efficient gene transfer and high resultant levels of expression. Integration of the gene in question to the cell's genome is permanent. Nevertheless, a retrovirus only infects cells undergoing mitosis; therefore, there is low transduction efficacy. Furthermore, given that introduction of the retrovirus to the cellular genome is permanent, it is also possible to permanently further mutate the already damaged genome (41).

The adenovirus is capable of infecting both dividing and quiescent cells with high efficacy. It can include up to 7.5 kilobytes of exogenous genetic material. Also, the adenovirus is not incorporated into a cell's genome, which avoids possible genomic mutations. However, resultant gene expression is transient, requiring repetitive treatments. It is also possible for the adenovirus to incite immune and inflammatory reactions (41). Even so, adenoviral vectors are currently employed in more than 70% of clinical gene therapy trials worldwide and show promising results in preclinical cancer treatment (17).

Recent data indicate, however, that most of the adenoviral vector clinical trials are in phase I, with only a minor percentage in phase III. This demonstrates that numerous obstacles may be related to the development of effective gene delivery systems and the urgent need for the development of new nonviral vectors (18).

Nanoparticles (NPs) have recently taken center stage as potential delivery vehicles since they can be designed to slip between intercellular spaces, enter cells or pass directly through biological barriers. NPs also protect encapsulated genes from potential enzymatic degradation, metabolism and filtration (19). However, since a non-viral vector must be nontoxic and biodegradable for the possibility of *in vivo* trials, current research has focused upon the development of biocompatible and biodegradable NPs.

One such particle is poly(lactic-CO-glycolic acid) or PLGA. PLGA NPs are approximately 200 nm in diameter (19). They are capable of crossing the endosomal barrier and effectively delivering their load to the cytoplasm via endo-lysosomal escape (Fig. 4.1). PLGA NPs are initially internalized into the cells by non-specific endocytosis. Once there, the anionic surface charge of the NPs changes to cationic due to the acidic pH of the endosome. This results in the escape of the NPs into the cytoplasm where the load is slowly released from the NP. The by-products of PLGA NPs are lactic and glycolic acids that are formed slowly and thus, easily metabolized in the body via the Krebs cycle prior to being eliminated (42). PLGA NPs, therefore, offer the advantages of safety.

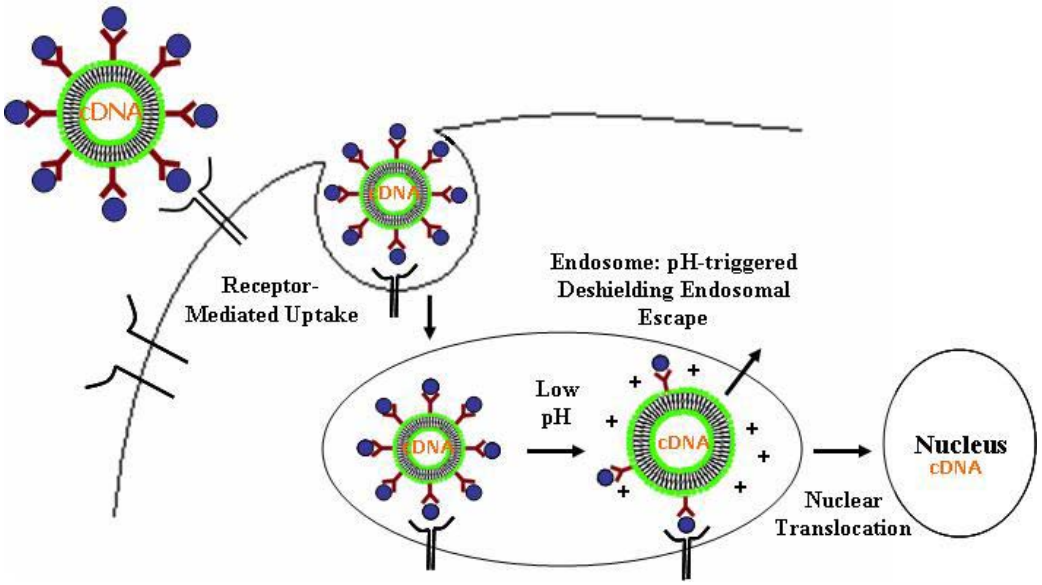

Figure 4.1 Schematic illustration of nanoparticle-based targeted gene expression.

(recreated from Boeckle and Wagner, AAPS J, 2006)

NPs can also be conjugated in order to specifically target the tissue in question (Fig. 4.1). For example, previous research has demonstrated effective nervous tissue targeting with surface conjugation using the transferrin ligand (43). In cancer, the EGF receptor has been used for tissue targeting (44). The ability to specifically target certain tissues is a valuable asset in cancer therapy as normal tissues remain unaffected. The only weakness of NP-based gene therapy is that gene expression is not as robust as that obtained from adenoviral-based gene delivery. However, due to the slow degradation of the NP, the encapsulated gene is slowly released. Therefore, NP-based gene delivery is capable of sustained expression. NPs are, therefore, suitable for gene delivery.

Chapter 5 - Viral Vector-Based AT_2 Receptor Gene Delivery

The research regarding the role of the overexpressed AT_2 receptor in lung tumorigenesis has two primary facets: (1) delivery of the receptor with an adenovirus as the carrier and (2) delivery of the receptor using PLGA nanoparticles as the carrier. The main goal is to demonstrate that gene therapy utilizing the AT_2 receptor is a viable therapeutic option in treating NSCLC. Furthermore, this research will demonstrate the ability of PLGA nanoparticles as vectors to specifically target and inhibit lung cancer.

Effect of Vector-Based AT_2 Receptor Overexpression on the Growth of Human Lung Cancer Cells

The A549 and H358 cell lines are typical examples of human lung adenocarcinoma and bronchioalveolar carcinoma (a subtype of adenocarcinoma), respectively. Furthermore, A549 and H358 exhibit negligible levels of the AT_2 receptor and as such, levels of AT_2 receptor expression can be controlled (31). Therefore, these cell lines are well-suited for examining the effects of AT_2 overexpression on lung cancer.

A549, H258 and BEAS-2B cells were transfected with either adenoviral AT_2 (Ad-AT_2) or adenoviral β-galactosidase (Ad-LacZ) at 50 multiplicity-of-infection (MOI) and cultured for 48 hours (see Chapter 9, Adenoviral Transfection). After subculturing, the resultant cell number was determined by hemocytometer 72 hours later. By examining Figure 5.1, it is apparent that Ad-AT_2 inhibited cell growth. It is also worthwhile to note that Ad-LacZ transfected cells also showed a slight decrease in cell number as compared to the non-transfected cells. As discussed in Chapter 3, the adenovirus itself can also have cytotoxic effects. Ad-LacZ allows elimination of these false positives. LacZ is a reporter gene that encodes for β-galactosidase. It is not present in the A549 and H358 genome and therefore serves as a good control.

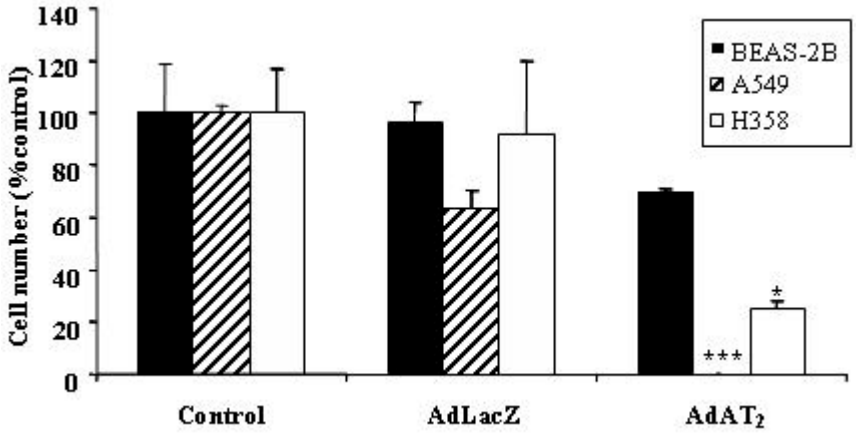

Figure 5.1 Effect of adenoviral vector-based AT_2 receptor overexpression on cell proliferation in A549, H358 (human lung cancers) and BEAS-2B (normal bronchial epithelial) cells.

Cells were treated with 50 MOI Ad-AT_2 or Ad-LacZ and cultured for 48 hrs, then subcultured for an additional 72 hrs. The viability of control cells was set to 100% and the survival relative to control is presented. Each value represents mean ± SD of three independent experiments. Statistical significance is indicated by *, $p < 0.05$; ***, $p < 0.001$. All p-values are as compared to the control.

H358 and A549 cells showed a 60% and 80% decrease in the number of live cells, respectively (Fig. 5.1). The cell morphology in Ad-AT_2 transfected cells was also strikingly different from Ad-LacZ transfected and non-transfected cells. The cells exhibited features typically characteristic of cell death such as round shape and detachment from the culture dish as early as 24 hours after subculture (Fig. 5.2).

The effects of AT_2 receptor overexpression were also examined in a normal human bronchial epithelial cell line, BEAS-2B. BEAS-2B cells did not show any significant decrease in cell number when transfected with Ad-AT_2 as compared to Ad-LacZ and the non-transfected controls (Fig. 5.1). Additionally, the cell morphology remained normal with no indicators of cell death such as blebbing or dish detachment (data not shown).

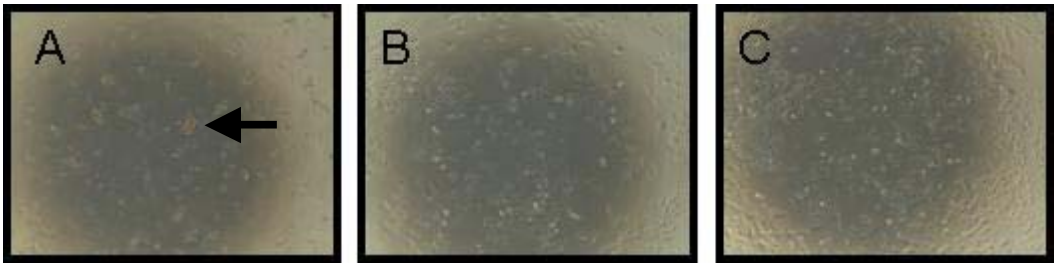

Figure 5.2 Effect of AT_2 receptor overexpression on A549 cell morphology.

Ad-AT_2 transfected A549 cells (A) exhibit features characteristic of cell death such as blebbing or dish detachment (indicated by arrow). Ad-LacZ (B) transfected and non-transfected (C) cells did not show such effects. Cells were treated with 50 MOI Ad-AT_2 or Ad-LacZ and cultured for 48 hrs, then subcultured for an additional 72 hrs. Images were captured after the 72 hr time point.

The decrease in cell growth of lung adenocarcinoma is also supported by the inhibition of DNA synthesis. Tritiated thymidine is commonly used to examine cell proliferation (see Chapter 9, [^{3}H]-Thymidine Uptake). The level of the resultant incorporation is detected by a beta scintillation counter and is proportional to DNA synthesis in the cell, which correlates to cell proliferation.

A549 cells were transfected as before (50 MOI) with Ad-AT_2 (see Chapter 9, Adenoviral transfection). AT_2 receptor overexpression in A549 cells decreased [^{3}H]-thymidine uptake, and therefore, DNA synthesis, by 80% as compared to the untreated control (Fig. 5.3).

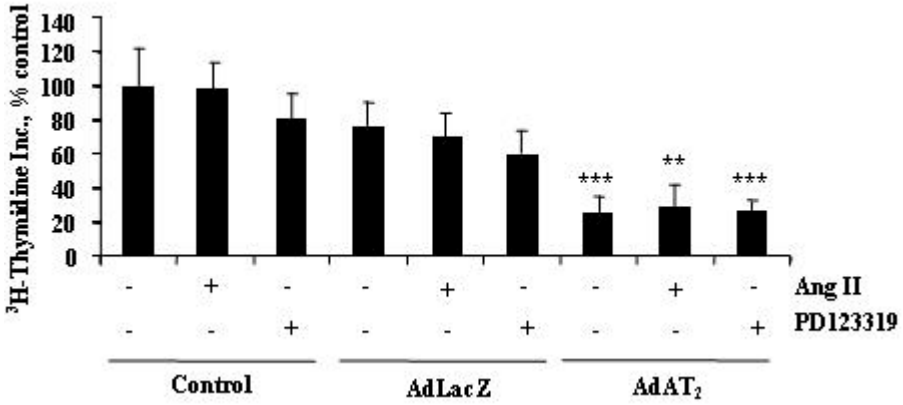

Figure 5.3 Effect of AT_2 receptor overexpression on DNA synthesis in A549 cells and its ligand-dependency.

Cells were pretreated with Ang II or PD123319 for 15 minutes, then transfected with 50 MOI Ad-AT_2 or Ad-LacZ and cultured for 48 hrs and then subcultured for an additional 72 hrs. The percentage of thymidine uptake is calculated and illustrated. Each value represents mean ± SD of three independent experiments. Statistical significance is indicated by **, $p < 0.01$; ***, $p < 0.001$. All p-values are as compared to the non-treated control.

The AT_2 receptor antagonist PD123319 (10^{-6} M) and the AT_2 receptor ligand Ang II (10^{-8} M) were used to link the decrease in cell proliferation and DNA synthesis to the AT_2 receptor. PD123319 should block the effects of the receptor-mediated functions whereas Ang II may further stimulate the effects. Interestingly, the inhibition of cell growth and DNA synthesis could not be blocked by PD123319 nor stimulated by Ang II. These effects are supported by previous research that the AT_2 receptor functions independent of its ligands (8). It has furthermore been shown that the AT_2 receptor is constitutively active and induces cell signaling on its own (45).

Therefore, in order to confirm that the inhibition of cell growth was indeed due to AT_2 receptor overexpression, real time PCR was used to examine AT_2 receptor mRNA expression (see Chapter 9, Real Time RT-PCR). Ad-LacZ transfected and non-transfected cells did not show any increased expression of the AT_2 receptor. However, Ad-AT_2 transfected cells showed a significant increase in receptor expression (Fig. 5.4). This indicates that AT_2 receptor expression is associated with the decrease in DNA synthesis and cell proliferation.

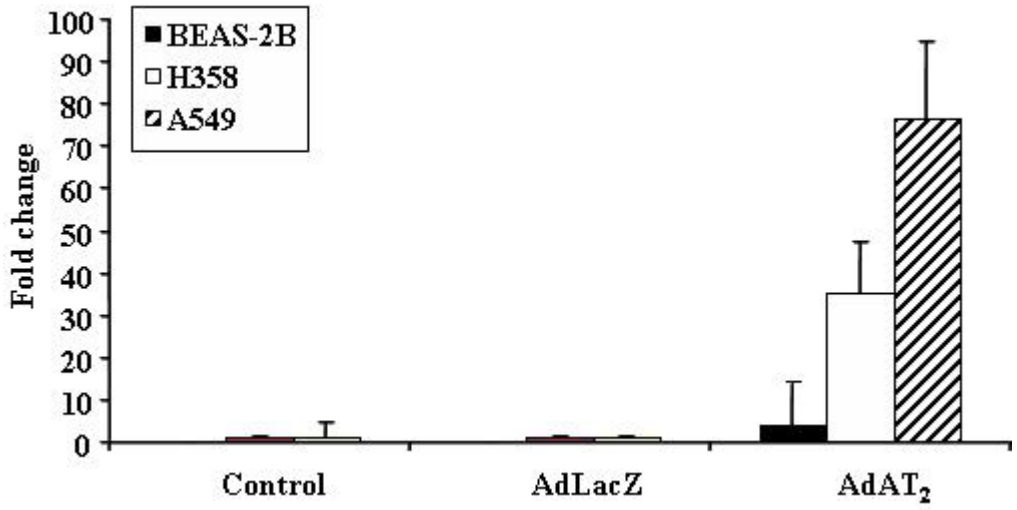

Figure 5.4 Adenoviral vector-based AT_2 receptor induced overexpression of the AT_2 receptor in BEAS-2B, H358 and A549 cells.

Cells were transfected with 50 MOI Ad-AT_2 or Ad-LacZ and cultured for 48 hrs. mRNA was collected using TRIzol reagent and expression levels were determined using real-time PCR on the Icycler from Biorad. Each value represents mean ± SD of three independent experiments.

The ability of AT_2 receptor overexpression in lung cancer cells to decrease cell proliferation and DNA synthesis suggests that the AT_2 receptor may have significant effects on lung cancer tumorigenicity.

A soft agar assay (Fig. 5.5) is a unique method to test anchorage independent growth of cancer cells *in vitro*. The growth process of normal epithelial cells is to grow in a monolayer and subsequently stop once they come into contact with other cells. Cancer cells, however, have aberrant expression of cell adhesion molecules (CAM) that alter their characteristics. Cancer cells do not stop growing once they touch other cells; rather, they begin to pile up on top of one another, forming colonies and eventually tumors. A soft agar assay provides a unique support system for cancer cells by allowing them to form colonies. Ad-AT_2 transfected A549 cells were grown in soft agar for 7-14 days, after which colony formation was assessed (see Chapter 9, Soft Agar Assay). Ad-AT_2 transfected cells showed a significant decrease in colony formation: on average 85.6% less colonies than A549 non-transfected and Ad-LacZ transfected cells (Fig. 5.6).

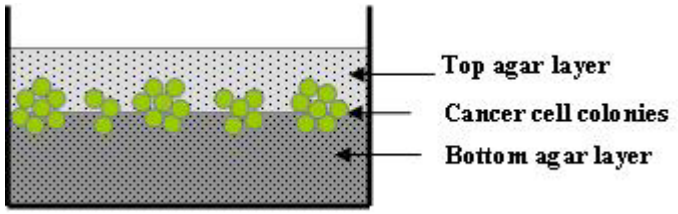

Figure 5.5 Schematic illustration of a soft agar assay

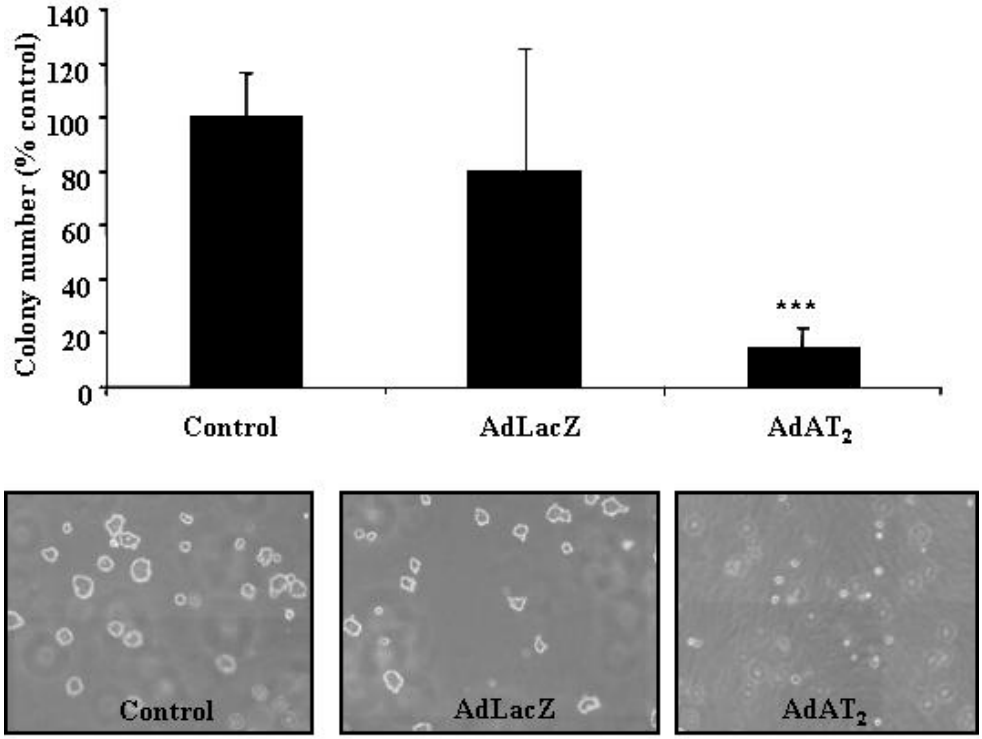

Figure 5.6 Effect of AT_2 receptor overexpression on A549 anchorage-independent cell growth.

Cells were transfected with 50 MOI Ad-AT_2 or Ad-LacZ and cultured for 48 hrs and then suspended in the top agar layer. All values are normalized to the number of colonies found in the non-transfected wells. Each value represents mean ± SD of three independent experiments. Statistical significance indicated by ***, $p < 0.001$. The p-value is as compared to the control.

These studies suggest that AT_2 receptor overexpression in NSCLC not only decreases DNA synthesis and cell proliferation, but it decreases the facility for anchorage-independent growth, a hallmark of tumorigenesis. Therefore, forcing AT_2 receptor expression in lung cancer cells may decrease tumorigenesis.

The Mechanism of AT_2-Induced Cell Death

Thus far, the inhibition of cell proliferation, DNA synthesis and anchorage-independent growth has been attributed to overexpression of the AT_2 receptor. However, previous research has indicated that the upregulation of the AT_2 receptor in serum-starved cells further signals for apoptosis, or programmed cell death (8). As such, the next step was to examine Ad-AT_2 transfected cells for the translocation of phospholipid phosphatidylserine (PS) to the outer surface of the plasma membrane, an early indicator of apoptosis. The protein annexin V has a

high binding affinity for PS. Therefore, when Annexin V is bound to the fluorochrome FITC, it allows simple flow-cytometric identification of early apoptotic cells (see Chapter 9, Annexin-V Assay). Five days after transfection with Ad-AT_2, it was found that 38% of the total cell population was apoptotic, whereas non-transfected cells and Ad-LacZ transfected cells only showed 2% and 3% apoptotic cells, respectively (Fig. 5.7).

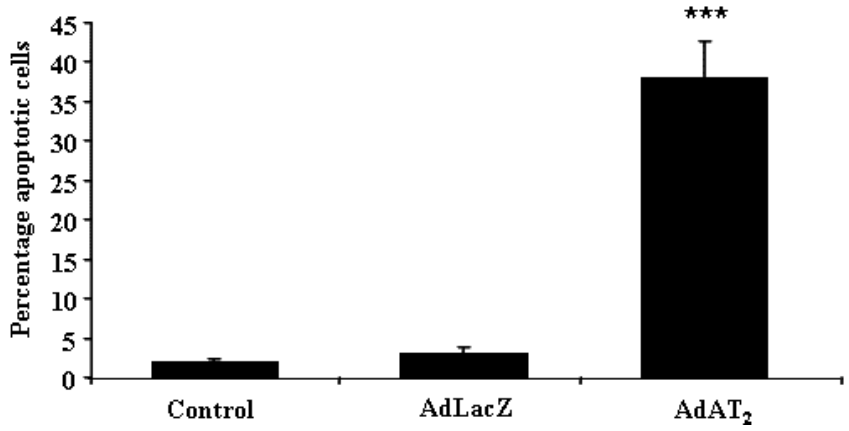

Figure 5.7 Effect of AT_2 receptor overexpression on apoptosis in A549 cells.

Cells were transfected with 50 MOI Ad-AT_2 or Ad-LacZ and cultured for 48 hrs, then subcultured for an additional 72 hrs. The percentage of apoptotic cells is as compared to the total cell population. Each value represents mean ± SD of three independent experiments. Statistical significance indicated by ***, $p < 0.001$. The p-value is as compared to the control.

The mechanism by which apoptosis is induced by the AT_2 receptor was also evaluated. A prime candidate is the caspase pathway, as the caspases are believed to be the central executioners of the apoptotic pathway. Therefore, Ad-AT_2 transfected cells were examined for caspase-3. Within 24 hrs, procaspase-3 showed a slight decrease in expression. By 72 hrs, procaspase-3 expression had decreased by 60% (Fig. 5.8). This is significant as the reduction in procaspase-3 expression indicates activation of the apoptosis pathway. It can therefore be concluded that AT_2 receptor overexpression induces apoptosis through caspase activation in lung cancer cells.

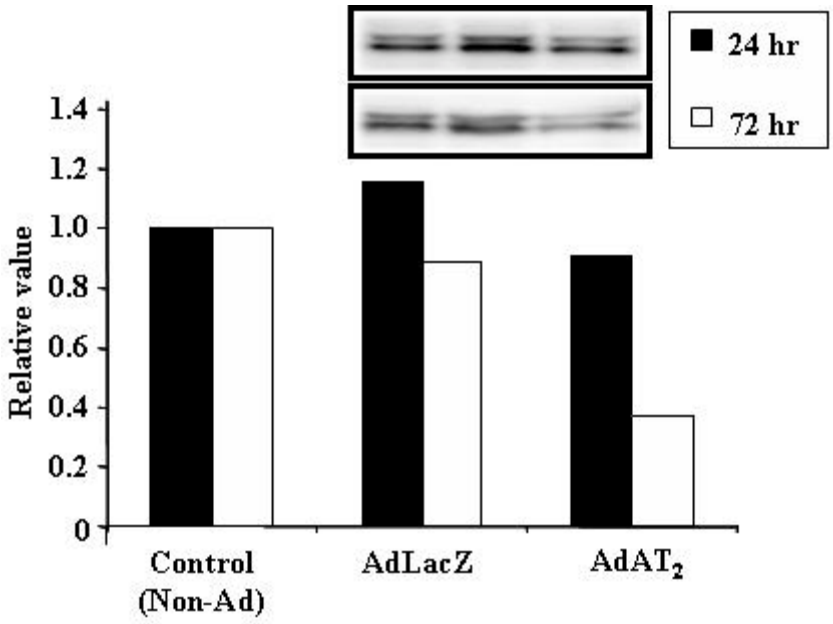

Figure 5.8 Effect of AT_2 receptor overexpression on the caspase pathway.

Cells transfected with 50 MOI Ad-AT_2 or Ad-LacZ for 24 and 72 hrs. Values are relative to the control band.

Chapter 6 - Nanoparticle-Based AT_2 Gene Delivery

Uptake Kinetics of AT_2 Receptor DNA Encapsulated PLGA Nanoparticles

Having examined the effect of AT_2 receptor overexpression using the adenoviral vector on multiple cell lines, the nanoparticle study focused on the A549 adenocarcinoma cell line.

AT2 receptor cDNA was isolated from plasmid bacterial growth (see Chapter 9, Plasmid cDNA Preparation), and then delivered to the University of Kansas (Lawrence) for encapsulation with PLGA nanoparticles. The PLGA particles used in this study were kindly provided by Dr. Cory Berkland at the University of Kansas (Lawrence). They are uniform in shape and approximately 200 nm in size (Fig. 6.2A). The particles have demonstrated excellent uptake kinetics in a variety of cell lines. A549 cells were incubated with the following three different types of nanoparticles (see Chapter 9, Nanoparticle Transfection):

(1) PLGA particles (Fig. 6.2A),

(2) Unconjugated AT_2 receptor encapsulated PLGA particles (Fig. 6.1A) or

(3) Ang II conjugated AT_2 receptor encapsulated PLGA particles (Fig. 6.1B).

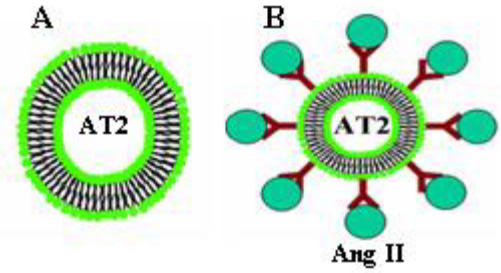

Figure 6.1 Schematic illustration of an unconjugated (A) and an Ang II conjugated (B) AT_2 receptor encapsulated PLGA nanoparticle.

After 15 minutes of incubation, the NPs were removed and cells were imaged using the Nikon Eclipse 80i fluorescence microscope at 15 minutes, 1 hour and 24 hrs. For this initial test, the NPs did not encapsulate the AT_2 receptor, but rather the fluorescent dye Coumarin-6. Therefore, for fluorescence microscope detection, 450 nm was used for excitation and a 500 nm long pass filter for emission. Within 15 mins, NPs conjugated with Ang II were uptaken by the

cell, as indicated by the clear outline of the cancer cell (Fig. 6.2A). Unconjugated nanoparticles, however, did not demonstrate such efficient uptake kinetics (Fig. 6.2B). Contrarily, if the cells are incubated with the NPs for 5 hrs, the unconjugated particles are uptaken by the cells (data not shown).

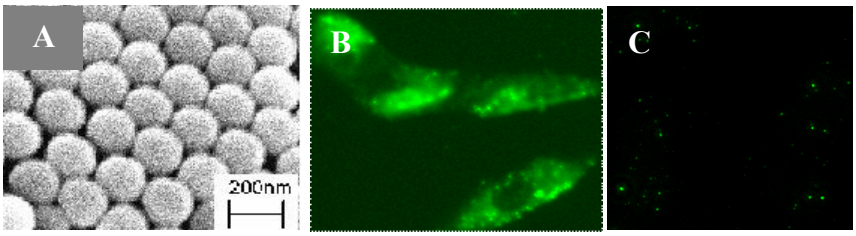

Figure 6.2 Internalization of PLGA nanoparticles.

Scanning electron microscope image of PLGA nanoparticles (A). Ang II conjugated particles (B) were uptaken in A549 cells in 15 minutes but unconjugated particles (C) were not effectively uptaken.

Effect of PLGA Nanoparticle-Based AT_2 Receptor Overexpression on the Growth of A549 Cells

Having demonstrated that the PLGA particles are effectively taken up in cancer cells, nanoparticle AT_2 gene delivery efficacy was next examined. Since these trials were done *in vitro*, the use of Ang II conjugated particles was unnecessary; therefore, cells were examined without nanoparticles, empty particles and AT_2 encapsulated particles.

Real time RT-PCR (see Chapter 9, Real Time RT-PCR) was used to examine the fold-change in AT_2 receptor gene expression, whereas the MTT assay (see Chapter 9, MTT Assay) determined cell viability.

A549 cells showed both a dose- and time-dependent response to AT_2 receptor encapsulated NPs. Three days after NP treatment, AT_2 receptor mRNA expression reached its maximum in cells incubated with AT_2 receptor encapsulated NPs. With 3 μg NPs, a 120-fold increase in AT_2 receptor mRNA expression was found (Fig. 6.3A). Higher doses achieved coordinating higher expressions; however, at doses higher than 3 μg, the empty nanoparticles began to exhibit cytotoxic effects on A549 cells.

A549 cell viability was similarly affected by increasing concentrations of AT_2 receptor encapsulated NPs. Twenty-four hours post NP treatment, A549 cell viability decreased dose-dependently. NP concentrations of 1 µg or less did not affect cell viability. At 3 µg NPs, cell viability decreased by 13%. 10 µg NPs reduced cell viability by 27.6% and 30 µg decreased cell viability by 38.5%. Three days after the NP treatment, slight decreases in cell viability were visible at concentrations less than 1 µg. With 1 µg NPs, cell viability dropped by 31.5%. Further reductions in cell viability were difficult to ascertain. By 10 days, however, A549 cell viability decreased by greater than 75% at a concentration of 1 µg NPs. However, higher concentrations of NPs did not further reduce A549 cell viability (Fig. 6.3B). Nevertheless, morphological observation indicated that 1 µg – 50 µg AT_2-NP treatment almost completely killed the cells. Accordingly, it is concluded that the AT_2-NP treatment is extremely effective in cancer cell death induction. Thus, it is appropriate to pursue an animal study with this novel method.

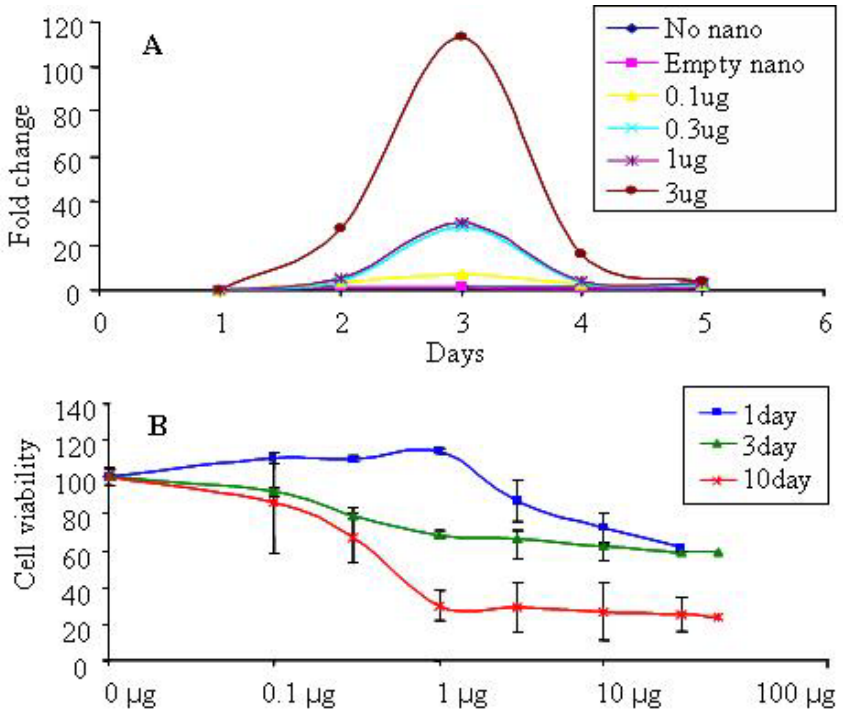

Figure 6.3 Effect of AT_2 receptor encapsulated PLGA nanoparticles on AT_2 receptor gene expression and cell viability in A549 cells.

Cells were incubated with nanoparticles for 5 hrs at varying concentrations as indicated in the figure. After removal of the particles, cells were cultured for 1, 2, 3, 4, 5 or 10 days. Gene expression (A) and cell viability (B) was evaluated by real time PCR and the MTT assay, respectively. Each value represents means ± SD of three independent experiments.

Chapter 7 - Future Work

Future experiments will evaluate (1) the nanoparticle-based gene delivery method *in vivo* and (2) the effect of AT_2 gene therapy on lung tumorigenesis in a mouse model.

First, distribution of the nanoparticles within the lung will be examined. Using a Microsprayer (Penn-Century) for intratracheal spray, rhodamine and/or Nile Red dye-loaded PLGA NPs will be administered. Because the solution will be applied directly to the lungs, it will be important to closely monitor the total fluid intake as greater than 0.02 mL induces suffocation and death in mice (46). At the specified time points, the mice will be sacrificed and the lungs removed and snap frozen for later examination. If the nanoparticles have diffused throughout the entire lung, the effect of AT_2 gene therapy will be examined. If not, other methods of nanoparticle administration will be explored.

Second, upon successful growth of A549 tumor xenografts in the lung, Ang II conjugated AT_2 encapsulated NPs through the above described intratracheal spray will be administered. We will use the following two mice groups: (1) control mice (no treatment) and (2) mice treated with the NP. In the *in vitro* studies, the appropriate quantity of AT_2 encapsulated NPs for cell death induction will be determined. The number of administrations necessary for effective treatment will also be determined.

After completion of the gene therapy regimen, all mice will be sacrificed and the resultant tumor sizes will be compared. Histopathological analysis of the tumors will be carried out with the assistance of the Histopathology Core Laboratory in the KSU College of Veterinary Medicine.

Chapter 8 - Discussion and Conclusions

In this thesis, the *in vitro* efficacy of the AT_2 receptor overexpression to induce apoptosis in A549 (human lung adenocarcinoma) and H358 (human bronchioalveolar carcinoma) cells but not in normal bronchial epithelial cells, BEAS-2B was demonstrated. The capability of both the replication-deficient adenovirus and the nanoparticles as gene delivery vehicles to lung cancer cells was also demonstrated.

This study first showed the ability of transfected Ad-AT_2 to induce cell death in lung cancer cells. A549 and H358 cells expressing AT_2 showed an 80% and 60% decrease, respectively, in live cells when compared to non-AT_2-receptor-expressing cells 5 days after the initial transfection. The Ad-AT_2 transfected cells also displayed features characteristic of cell death such as a round shape and subsequent detachment from the culture dish 72 hrs after transfection. This effect lasted for up to ten days. A recent study indicates that nuclear DNA condensation and fragmentation began approximately 24 hrs after AT_2 receptor overexpression (8). Therefore, visual confirmation of cell death 72 hrs after transfection is appropriate.

BEAS-2B cells, normal bronchial epithelium, did not demonstrate any significant decrease in cell viability when transfected with Ad-AT_2. The minimal decrease seen in cells transfected with Ad-LacZ can be attributed to adenovirus-dependent nonspecific alterations. These results suggest that the AT_2 receptor specifically targets lung carcinoma cells with minimal damage to normal tissues. Furthermore, the effects of the AT_2 receptor are not cell line specific as both the A549 and H358 cell lines were affected significantly by AT_2 receptor overexpression.

It appears that basal physiological expression levels of the AT_2 receptor do not affect cell viability although the AT_2 receptor expression levels in non-transfected A549 and H358 cells are significantly low. This is supported by the PC12W cell line, which naturally expresses the AT_2 receptor. Serum-depletion increases AT_2 receptor expression 5-fold and then decreases cell viability (45, 47). Although the overexpressed AT_2 receptor is constitutively active, further studies have indicated that cell death was induced only when the receptor underwent homo-oligomerization (45).

[^{3}H]-thymidine, or tritiated thymidine, then revealed the inhibition of DNA synthesis by the AT_2 receptor overexpression in A549 cells. Thymidine uptake decreased by 80% in Ad-AT_2

transfected cells. DNA synthesis was neither inhibited nor further stimulated when pretreated with the AT_2 receptor ligand, Ang II, or the AT_2 receptor-specific antagonist, PD123319. The AT_2 receptor has also been shown to exert an inhibitory effect on DNA synthesis in vascular smooth muscle cells (48) and fibroblasts (49).

Forced expression of the receptor significantly reduced cell proliferation and DNA synthesis in lung cancer cells and was reconfirmed in this study as apoptosis. AT_2 receptor expressing A549 cells showed a 15% increase in apoptotic cells as compared to non-transfected and LacZ expressing cells. Activation of the receptor via the ligand Ang II is not required as Muira et al state that the induction of apoptosis is an intrinsic property of the AT_2 receptor. They propose that Ang II activation is not essential for apoptosis and that apoptosis cannot be blocked by PD 123319. Transfected AT_2 receptors also activate p38 MAPK-mediated apoptotic signaling and the activation of the cell death-promoting proteases (8, 45). This is seen in this study's and other studies' detection of procaspase-3 expression reduction in AT_2 receptor expressing cells (8).

A tumor colony assay further evaluated AT_2 overexpression effects on A549 cancer cell growth. AT_2 overexpression significantly reduced the formation of colonies, by more than 80%. These results indicate that AT_2 receptor overexpression efficiently inhibits colony formation. Furthermore, transfected AT_2 receptor has been demonstrated to halt progression in pheochromocytoma, a tumor of the adrenal medulla (50).

PLGA NPs as a gene delivery system *in vitro* were also evaluated. AT_2 encapsulated NPs were effectively taken up into A549 cells. When conjugated with Ang II, NP internalization was found as early as 15 minutes after incubating the cell culture with the particle. Unconjugated nanoparticles did not demonstrate such effective uptake kinetics. However, when the cells are incubated with the NPs for 5 hours, the AT_2 encapsulated PLGA particles are efficiently uptaken. For the *in vitro* studies, accelerated uptake kinetics are non-essential. However, the quick uptake seen in the Ang II conjugated particles is valuable for future *in vivo* studies.

When incubated with AT_2 encapsulated NPs, A549 cells demonstrated a time- and dose-dependent response. The resultant transfection of the AT_2 gene was robust and effective in sustained gene expression. AT_2 receptor expression was detected as early as 2 days after transfection with the highest expression at 3 days. Accordingly, cell viability began to decrease significantly by day 3 and lasted through day 10.

These findings indicate that overexpression of the AT_2 gene is a potential treatment for lung cancer with minimal side effects. This is the first study describing AT_2 receptor overxpression as potential gene therapy for non-small cell lung cancer. However, further in-depth investigations are essential to elucidate the efficacy of the AT_2 in *in vivo* animal models.

Chapter 9 - Materials and Methods

Viral Vectors

To generate a recombinant replication-deficient adenovirus expressing AT_2 receptor, EcoRI-XbaI fragment of pCS027 mouse AT_2 receptor cDNA construct (generous gift from Dr. Inagami, Vanderbilt University, Department of Biochemistry, Nashville, TN) was subcloned into a recombinant adenovirus shuttle vector pACCMVpLpA which has deletions in the *E1A*, *E1B* and *E3* genes (51). The pACCMVpLpA-AT_2 plasmid was transfected in HEK93 cells, purified, expanded and titered by detection of the formation of visible plaques in HEK293 monolayer. The replication-deficient adenovirus vector expressing Lac Z (β-galactosidase) was provided by Dr. Myers (Vanderbilt University, Department of Medicine, Nashville, TN).

Cell Cultures.

Human lung adenocarcinoma cell line A549, bronchioalveolar carcinoma H358 and normal human bronchial epithelium BEAS-2B were purchased from American Type Culture Cell (Manassas, VA). Cell lines A549, H358 and BEAS-2B were cultured with Ham's F-12, RPMI-1640, and LHC-9 (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 100 units / mL penicillin and 100 μg / mL streptomycin (Invitrogen, Carlsbad, CA), respectively. The cells were incubated in 5% CO_2 humidified at 37°C.

Real Time RT-PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from the cells that were gene-transduced. Genomic and complementary DNA was removed using RQ1 RNase-free DNase (Promega, Madison, WI) according to the manufacturer's instructions. Real-Time PCR was carried out using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) and the reactions were conducted on the real-time PCR detection system iCycler (Bio-Rad, Hercules, CA). The results were quantified as C_t values, where C_t is defined as the threshold cycle of PCR at which the amplified product is first detected and defined as relative gene expression (the ratio of target/control). The AT_2 primers were 5' – AGC CAA GGC CAG ATT GAA GA – 3' (forward) and 5' – GCC ACC AGC AGA AAC ATT ACC – 3' (reverse). The LacZ primers

were 5' – CCC AAC TTA ATC GCC TTG CA – 3' (forward) and 5' – GCG GGC CTC TTC GCT ATT – 3' (reverse). The 18S ribosome RNA primers were 5' – TCG CTC CAC CAA CTA AGA AC – 3' (forward) and 5' – GAG GTT CGA AGA CGA TCA GA – 3' (reverse).

mRNA Extraction

For all steps, clean gloves are an absolute must. Until step #6, all steps must be performed in the downdraft hood. For step #6 and subsequent steps, use RNaseZap Wipes to cleanse gloves and use the counter indicated RNase-free. This counter must be cleaned prior to each use.

1) Aspirate the medium from the well and rinse each well twice with PBS.
2) Add the TRIzol reagent. (0.5 mL to a 35 mm dish, 1.5 mL to a 10 cm dish)
3) Cell scrape using a 24 cm cell scraper from TPP and collect the cell suspension into a 1.5 mL centrifuge tube.
4) Add the same amount of TRIzol reagent to the dish and cell scrape again. Collect as much solution as possible to the 1.5 mL centrifuge tube.
5) Incubate for 5 min at RT.
6) At this point, all steps may be completed outside the downdraft hood. Add 0.2 mL chloroform per 1 mL of TRIzol. Close the cap and shake the tube vigorously.
7) Incubate for 10 min at RT.
8) Centrifuge for 15 min at 14000 rpm at 4 °C in the Eppendorf Centrifuge 5145C. *If you touch anything not on the RNase-free counter with your gloves, new must be used.*
9) Collect 400 µL of the upper phase (aqueous phase) and transfer it to a new 1.5 mL centrifuge tube. In order to avoid collecting the DNA trapped in the interphase and the protein trapped in the pink phase, use a 200 µL pipette and pipet very slowly.
10) Add 400 µL of isopropanol and 1 µL glycogen to the 400 µL RNA mixture collected in the previous step. Mix gently and incubate 10 min at RT.
11) Centrifuge for 20 min at 14000 rpm in the Hermle Z 180M centrifuge (model # Z18M-24).
12) Remove the supernatant using the 1 mL pipette. The resultant pellet can be nearly invisible; therefore, be extremely careful not to remove the pellet as this is the mRNA.
13) Add 1 mL of 70% EtOH and mix gently. To mix, close the cap and gently invert the tube until the pellet floats freely in the EtOH.

14) Centrifuge for 5 min at 14000 rpm in the Hermle Z 180M centrifuge (model # Z18M-24).
15) Repeat steps #12 - #14 twice for a total of three times.
16) After the third centrifugation, remove the supernatant and then invert the tube, with cap open. Place the tube on a Kim Wipe to dry for 5 min.
17) Add 100 μL DNase-free water.
18) Incubate the RNA for 10 min at 55 °C in the Precision stainless steel water bath (model #183).
19) Measure the mRNA concentration using the Nanodrop 8000 (model #ND-8000).

Adenoviral Transfection

At greater than 90% confluency, cells were incubated at 37°C for 1 hr in a small amount of DMEM containing 5% FBS and adenoviral vectors, and shaken lightly every fifteen minutes. After 1 hr, additional DMEM containing 5% FBS was added and then incubated at 37°C, 5% CO_2 for a further 48 hrs. A multiplicity of infection (MOI) of 50 was used for all transfections.

Adenoviral Transfection

Note: When expanding the virus for further use, use the HEK293 cell line. Otherwise, this protocol is valid for all cell lines.

(1) Remove medium from the well.
(2) Rinse twice with 1x PBS.
(3) Add 5% serum (+) medium to the well (0.5 mL per 25 cm^2).
(4) Add the appropriate volume of virus for the necessary MOI.
(5) Gently spread the mixture so that it covers the entire well.
(6) Incubate for 15 minutes at 37°C and 5% CO_2 in the Fisher Scientific Isotemp incubator.
(7) Repeat steps #5 and #6 three times (for a total of four times).
(8) Add 5% serum (+) medium to each well (4.5 mL per 25 cm^2).

Adenoviral Collection

Note: Once the cells have been transfected, wait 3-4 days or until 50% of the cells have lifted.

(1) Rinse the dish to lift all remaining cells and collect the cell / media mixture to a 50 mL tube.
(2) Freeze in the -80°C freezer for 1 hr.

(3) Thaw quickly in the Precision stainless steel water bath (model #183) at 37°C.
(4) Sonocate (Bransonic 52) three times for 1 min, with 5 min intervals.
(5) Spin down for 3 min at 1000 rpm in the Beckman GS-6KR centrifuge.
(6) Collect the supernatant to a new 50 mL tube.
(7) Repeat steps #2-6 twice for a total of three times.
(8) After the final spin, aliquot the supernatant to 1 mL cryovials.

Plasmid cDNA Preparation

Recombinant plasmids were isolated from colonies using Quantum Prep plasmid maxiprep kit (Bio-Rad laboratories, Hercules, CA, USA). Insertion of the PCR product into the plasmid was confirmed by restriction endonuclease digestion with *BamH*1 and *xho1* and subsequent gel electrophoresis. Detailed instructions can be found in the instruction booklet provided with the kit.

Nanoparticle transfection

PLGA nanoparticles encapsulated with AT_2 were generated by Dr. Cory Berkland at the University of Kansas. At 50% confluency, cells were incubated at 37°C for various time durations with serum(-) media and various concentrations of nanoparticles. Briefly, the media was removed from the well. The cells were then rinsed twice with PBS. The cells were then incubated at 37°C and 5% CO_2 for 5 hrs with the nanoparticles in serum(-) media. The supernatant was then removed and fresh media containing 10% FBS was added to the well.

(1) Remove media using a 1 mL pipette. *Do not use the asipirator to avoid lifting the cells from the dish.*
(2) Rinse each well twice with PBS. *Do not use the asipirator to avoid lifting the cells from the dish.*
(3) Add the appropriate concentration of NPs in serum(-) media.
(4) Incubate at 37°C, 5% CO_2 for 5 hrs in the Fisher Scientific Isotemp incubator.
(5) Remove the nanoparticle supernatant. *Do not use the asipirator to avoid lifting the cells from the dish*.
(6) Add 10% serum (+) media to the wells

MTT Assay

Cell viability was determined by a direct cell count of live cells. Cells were seeded at $0.3x10^6$ cells per well in a 6-well plate and allowed to grow to 90% confluency. For adenoviral overexpression, cells were treated with Ad-AT_2 and incubated for an additional 48 hrs. At the end of incubation cells were lifted and subcultured at a density of $1x10^4$ cells per well. The resultant cell number was evaluated at specific time points.

For nanoparticle-mediated AT_2 receptor overexpression, cells were seeded at 2500 cells per well in a 96 well plate and incubated 24 hrs for attachment. Media was then changed to media containing AT_2 encapsulated nanoparticles and incubated for various time points. Four hours prior to completion of incubation time, 10 μL of MTT solution (5 mg/mL) was added. After completion of the incubation, 100 μL of solubilization buffer (Isopropanol with 0.1N HCl) was added and then incubated at room temperature overnight. Absorbance was read at 550 nm on the Molecular Devices Spectramax 190 plate reader.

(1) Seed 2500 cells/well in a 96 well plate and incubate 24 hrs for attachment.
(2) Induce gene expression (see **Chapter 9, Nanoparticle Transfection**).
(3) Four hours prior to completion of time point, add 10 μL MTT solution to each well. Incubate at 37°C, 5% CO_2 for the remaining time.
(4) After completion of the incubation time, add 100 μL solubilization buffer (isopropanol with 0.1 N HCl) and keep at room temperature overnight.
(5) Shake plate using the AUTOMIX option on the Molecular Devices Spectramax 190 plate reader and read the absorbance at 550 nm and 630 nm.

[^{3}H]-Thymidine Uptake

Cell proliferation was examined by measuring DNA synthesis using tritiated thymidine ([^{3}H]-thymidine) uptake. Two million cells were cultured in 6 cm culture dishes and incubated 24 hrs for attachment. After 24 hrs, cells were transfected with Ad-LacZ or Ad-AT_2 (50 MOI) for 48 hrs. Cells were subcultured at a density of $4x10^4$ cells/well in a 24-well plate in 10% FBS media for 22 hrs and treated with Ang II (10^{-8} M) and PD123319 (10^{-6} M). Cells were pulsed for the

remaining 2 hrs with [^{3}H]-thymidine (2 μCi/ well). [^{3}H]-thymidine incorporation was analyzed by liquid scintillation counting using the Packard liquid scintillation counter Tri-Carb 2100TR.

(1) In three 6 cm dishes, plate two million cells in media containing 10% FBS.
(2) Incubate 24 hours for attachment.
(3) Transfect one dish with 50 MOI Ad-AT_2 and one dish with 50 MOI Ad-LacZ (see **Chapter 9**, **Adenoviral Transfection**).
(4) Incubate at 37°C, 5% CO_2 for 48 hrs in the Fisher Scientific Isotemp.
(5) Subculture each dish and reseed in a 24-well at a density of $4x10^4$ cells/well. Four wells should be the non-transfected control cells, four wells the Ad-LacZ transfected cells and twelve wells should be the Ad-AT_2 transfected cells. Of those twelve, four should be treated with Ang II (10^{-8} M) and four treated with PD123319 (10^{-6} M).
(6) Incubate at 37°C, 5% CO_2 for 24 hrs in the Fisher Scientific Isotemp.
(7) Two hours prior to completion of time point, add 2 μCi / 10 μL to each well and incubate for two hours
(8) Remove the culture media by aspiration.
(9) Fix the cells with 1 mL 10% TCA for 30 minutes.
(10) Wash the cells twice with 1 mL 10% TCA and aspirate all liquid from the wells.
(11) Add 0.5 mL 0.2N NaOH solution to all wells.
(12) Incubate the plate for 20-30 minutes at 37°C (without CO_2).
(13) Pipet 0.25 mL cell lysate into a counting vial containing 5 mL liquid scintillation cocktail.
(14) Count radioactivity using the Packard liquid scintillation counter Tri-Carb 2100TR.

Annexin-V Assay

Apoptosis was determined using an annexin V-fluorescein isothiocyanate/ propidium iodide (PI) kit according to the manufacturer's instructions (BD Pharmingen, San Jose, CA). Briefly, cells were washed with cold PBS, and resuspended in 1X Binding Buffer (10 mM HEPEN, pH 7.4, 140 mM NaCl and 2.5 mM CaCl2) at a concentration of $1x10^5$ - $5x10^5$ cells/mL. Cells were incubated at room temperature with 5 μL each of annexin V-fluorescein isothiocyanate and PI

for 5 minutes, and analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

(1) In three 6 cm dishes, plate two million cells in media containing 10% FBS.
(2) Incubate 24 hours for attachment.
(3) Transfect one dish with 50 MOI Ad-AT_2 and one dish with 50 MOI Ad-LacZ (see **Chapter 9**, **Adenoviral Transfection**).
(4) Incubate at 37°C, 5% CO_2 for 48 hrs in the Fisher Scientific Isotemp.
(5) Subculture and reseed at $0.3x10^5$ in a 6-well plate. Two wells should be the non-transfected control cells, two wells the Ad-LacZ transfected cells and two wells should be the Ad-AT_2 transfected cells.
(6) Incubate at 37°C, 5% CO_2 for 72 hrs in the Fisher Scientific Isotemp.
(7) Collect $1x10^5$ - $5x10^5$ cells by centrifugation at 1000rpm in the Beckman GS-6KR centrifuge.
(8) Rinse once with PBS.
(9) Resuspend cells in 500 µL of 1X Binding Buffer
(10) Add 5 µL of Annexin V-FITC and 5 µL of PI.
(11) Incubate at room temperature for 5 minutes in the dark.
(12) Analyze samples by flow cytometry on the FACS Calibur dual laser flow cytometer (Becton Dickinson, San Jose, CA).

Soft Agar Assay

Two million cells were cultured in 6 cm culture dishes and incubated 24 hrs for attachment. After 24 hrs, cells were transfected with Ad-LacZ or Ad-AT_2 (50 MOI) for 48 hrs. One million cells were suspended in the top layer of 0.4% agar in Ham's F-12 medium. The cell suspension was then overlaid onto the bottom layer of 0.8% agar in Ham's F-12 medium in a six-well plate in triplicate. The number of colonies with over 50 µm diameter was counted on day 7 and day 10 using an automated counter equipped with an inverted microscope at a 10x magnification (Hitschfel Instruments Inc., St. Louis, MO).

Western Blot Analysis

Total cellular protein was prepared using lysis buffer (1% TritonX-100, 0.1% SDS 0.25M sucrose, 1mM EDTA, 30mM Tris-HCl (pH 8.0)) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Protein samples were separated by a 12% SDS-PAGE gel, electrotransfered onto nitrocellulose membrane (Amersham Bioscience), and blocked with 5% nonfat dry milk in 0.1% Tween20 in TBS (TBST) 1 hour at room temperature. The membranes were washed and incubated with an anti-caspase-3 (Cell Signaling Technology, Inc., Danvers, MA) at a 1:500 – 1:4000 dilution with 5% nonfat dry milk in TBST overnight at 4°C and then with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences). The protein expression signal was detected with Pierce SuperSignal Western Blotting substrate (Pierce, Rockford, IL). GAPDH was used as the loading control of sample by reprobing with an anti-GAPDH antibody at a 1:8000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

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Appendix A - List of Acronyms

ACE	Angiotensin I-converting enzyme
Ad	Adenoviral vector
Ang II	Angiotensin II
AT_1	Angiotensin II Type 1 receptor
AT_2	Angiotensin II Type 2 receptor
CAM	Cell adhesion molecules
cDDP	Cisplatin
cDNA	Complementary deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EtOH	Ethanol
gDNA	Genomic deoxyribonucleic acid
IAP	Inhibitors of apoptosis protein
IGFR	Insulin-like growth factor receptor
IRF	Interferon regulatory factor
JNK	Jun N-terminal kinase
LacZ	β-galactosidase
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of infection
mRNA	Messenger ribosomal nucleic acid
MYO18B	Myosin 18B
NaOH	Sodium hydroxide
NCI	National Cancer Institute
NP	Nanoparticle
NSCLC	Non small cell lung cancer
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction

PDGFR	Platelet-derived growth factor
PLGA	poly(DL-lactide-*co*-glycolide
PS	Phospholipid phosphatidylserine
RT	room temperature
SCLC	Small cell lung cancer
STAT	Signal Transducers and Activators of Transcription protein
VEGF	Vascular endothelial growth factor