Actinomycin D and Telmisartan Combination Therapy Targets Lung Cancer Stem Cells

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Actinomycin D and Telmisartan Combination Therapy Targets

Lung Cancer Stem Cells

by

Ryan Green

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Medicine
College of Medicine
University of South Florida

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Keywords: Chemotherapy, 3D culture, Drug Synergy

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Abbreviations

2D Two dimensional
3D Three dimensional
ABCG2 ATP binding cassette sub family G member 2
AD Actinomycin D
ALDH Aldehyde Dehydrogenase Activity
ALK Anaplastic lymphoma kinase
AML Acute myeloid leukemia
ANP Atrial natriuretic peptide
APC Adenomatous polyposis coli
APC-Cy7 Allophycocyanin-Cyanine7
ATCC American Type Culture Collection
ATP Adenosine Tri-phosphate
BD Becton Dickenson
Casp-3 Caspase-3
CD133 Cluster of Differentiation 133 (prominin-1)
CD24 Cluster of Differentiation 24
CD26 Cluster of Differentiation 26 (dipeptidyl-peptidase-4)
CD44 Cluster of Differentiation 44 (phagocytic glycoprotein-1)
cGMP Cyclic guanine monophosphate
CK1α Casein kinase 1α
CM-H2DCFDA Chloro-methyl- 2',7'-dichlorodihydrofluorescein diacetate
CRISPR Clustered regularly interspaced short palindromic repeats
CSC Cancer stem cell
CXCL12 C-X-C motif chemokine 12
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DEAB</td>
<td>N,N-diethylaminobenzaldehyde</td>
</tr>
<tr>
<td>DKK</td>
<td>Dickkopf-related protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dvl</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast derived growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein-isothiocyanate</td>
</tr>
<tr>
<td>FZD</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>gLuc</td>
<td>Gaussia luciferase</td>
</tr>
<tr>
<td>GpC</td>
<td>Cytosine-guanine</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>Lgr5</td>
<td>Leucine-rich repeat-containing G-protein coupled receptor 5</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>LLC1</td>
<td>Lewis Lung Carcinoma</td>
</tr>
<tr>
<td>LRP6</td>
<td>Low-density lipoprotein receptor-related protein 6</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mPEG</td>
<td>Methoxy-polyethylene glycol</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cell</td>
</tr>
<tr>
<td>NOD SCIID</td>
<td>Non-obese diabetic severe combined immunodeficiency</td>
</tr>
<tr>
<td>Nos</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NPRA</td>
<td>Natriuretic peptide receptor A</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD SCIID gamma</td>
</tr>
<tr>
<td>Oct-4</td>
<td>(octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly lactic acid</td>
</tr>
<tr>
<td>PLAT</td>
<td>Plasminogen Activator, Tissue Type</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly lactic co-glycolic acid</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>ROR2</td>
<td>Receptor Tyrosine Kinase Like Orphan Receptor 2</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small Cell Lung Cancer</td>
</tr>
<tr>
<td>Sox2</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>Src</td>
<td>Proto-oncogene tyrosine-protein kinase Src</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TCF</td>
<td>Transcription factor (TCF/LEF family)</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor Micro-environment</td>
</tr>
<tr>
<td>TS</td>
<td>Telmisartan</td>
</tr>
<tr>
<td>U.S.</td>
<td>United States</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless/Integrated</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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Abstract

The failure of lung cancer treatments has been attributed partly to the development of drug resistance, however the underlying cellular and molecular mechanisms are poorly understood. It has been suggested that a very small group of specific cells within the heterogeneous tumors, cancer initiating stem cells (CSC), develop resistance to treatment, survive and later initiate the growth of new tumors. Due to their pivotal role in maintenance and relapse of tumors following the acquisition of drug resistance, we reasoned that novel drugs targeting cancer cells and CSC might provide the most effective treatments, if not a cure. To this end, we reported a polymeric nanofiber scaffold on which tumor cells develop into tumor organoids termed “tumoroids” that resemble in vivo tumors. Herein we report that lung cancer cells grown on the scaffold acquire CSC properties (aldehyde dehydrogenase (ALDH) activity, sphere formation, and tumor initiation). We have identified two key pathways that regulate this expansion namely Natriuretic Peptide Receptor A signaling (NPRA) and Wnt/β-catenin signaling. Screening of an NCI Diversity set identified a lead candidate drug targeting CSC, namely Actinomycin D (AD). AD is a well-studied anti-cancer drug, however it is known to have several drawbacks including high clinical toxicity and the development of resistance. In order to overcome these drawbacks, we tested AD treatment in combination with the angiotensin receptor antagonist, Telmisartan (TS) because it has been reported to reduce fibrosis in tumors allowing them to be more permeable to drugs. We have found that this novel combination treatment is effective in blocking CSC
enrichment in the polymeric nanofiber scaffold model. Furthermore, we demonstrate the effectiveness and synergistic action of the combination treatment in both an in vivo syngeneic mouse model and xenograft model revealing its ability to reduce tumor burden. We also provide evidence that β-catenin activation is at least partially responsible for the increase in CSC seen in scaffold culture and that the combination treatment reduces tumor burden in part by inhibiting Wnt/β-catenin pathway. This work establishes the utility of the scaffold-inspired tumoroids as a model system capable of enriching CSC in vitro or ex vivo for targeted drug screening and personalized medicine. It also identifies a promising novel treatment targeting CSC that, with further study, may be useful to improve therapeutic outcomes for lung cancer.
Chapter 1
Introduction

1.1 Objective

The objective of this dissertation is to characterize the expansion of lung cancer stem cells (CSCs) in a novel 3D cell culture environment and explore the molecular mechanisms by which 1) The 3D cell culture environment contributes to the expansion of CSCs and 2) A novel combination therapy is able to target and eradicate lung CSCs.

1.2 Motivation

The motivation for this work is to create an in vitro model to expand the rare CSC population in solid tumors, thus facilitating study of their properties and the screening of compounds for the ability to inhibit their growth. Using our model we have identified a combination therapy that is able to inhibit the growth of CSCs and reduce tumor burden. The work presented herein will describe how this treatment modulates the Wnt/β-catenin signaling pathway in cancer cells to decrease their self-renewal and proliferation ability, ultimately resulting in cell death by apoptosis.

1.3 Scope The scope of this project is to achieve a better understanding of the growth of CSCs in the tumor microenvironment and to identify therapeutic interventions to stop their growth through the use of a novel 3D in vitro model. These results will be
confirmed using in vivo models in order to validate the in vitro findings and confirm the utility of the novel model system. Lung cancer is currently the world’s deadliest cancer and new treatments are desperately needed to improve patient care. In the future, the findings presented herein may be further developed into such a treatment by revealing mechanisms to target and eradicate tumor initiating CSCs.

1.4 Overview

Chapter 2 will introduce background information on CSCs describing their discovery, role in the tumor microenvironment, identifying biomarkers, models currently used to study them, and efforts to target CSCs in the clinic. Chapter 3 will describe in detail, all experimental designs and methods used. Chapter 4 will explain how a novel 3D cell culture environment was used to promote stem-ness in lung cancer cell lines in vitro. Chapter 5 will explain how this in vitro model can be used to study signaling pathways influencing the growth of CSCs, with a focus on the natriuretic peptide receptor A pathway. Chapter 6 will introduce a novel combination treatment and characterize its effects in the in vitro 3D CSC model as well as in vivo mouse models. Chapter 7 will describe the molecular mechanisms responsible for the treatment’s effects, focusing on the Wnt/β-catenin pathway. Finally, chapter 8 will summarize all results of this study and comment on its contributions to the field of CSC research and its implications for development of more effective cancer treatments.
2.1 Clinical Significance of Lung Cancer and Unmet Need for Treatment

Lung cancer is currently the second most common type of cancer in men and women (following only prostate cancer in men and breast cancer in women) [1]. However, lung cancer is by far the deadliest type of cancer in both men and women causing over 154,000 deaths in 2018 in the U.S. alone [2]. This represents approximately 25% of all cancer deaths. The five-year survival rate for patients with lung cancer is one of the lowest of any cancer at 18.6%, while the overall five-year survival for cancer patients is 66.9% [3]. Lung cancer is typically divided into two main morphological categories; small cell (SCLC) and non-small cell (NSCLC). Non-small cell is by far the most common type accounting for 80% - 85% of all lung cancer cases, therefore this project will focus on models of NSCLC with possible future application to SCLC and other cancer types. NSCLC is further divided into three main sub-types, adenocarcinoma (currently 40% of cases), squamous cell carcinoma (30%), and large cell carcinoma (15%) with the remaining 15% made up of many rare sub-types[4]. While smoking is the largest risk factor for both small cell and non-small cell lung cancer, age should also be considered as the majority of diagnoses are made in patients over 65 and very rarely in patients younger than 45.
Standard of care treatments for lung cancer vary depending on sub-type and stage at the time of diagnosis. There are many options available including surgery, radiation, chemotherapy, targeted therapies, immunotherapies, and combinations of the above. Despite this, over half of lung cancer patients die within one year of diagnosis. This high mortality rate is largely due to the development of drug and radio-resistance within tumors during treatment. For example, in recent years many drugs targeting common driver mutations in lung cancer such as those in the epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), MET proto-oncogene receptor tyrosine kinase (MET), and B-Raf proto-oncogene serine/threonine kinase (BRAF) genes have been FDA approved and used as personalized second line (after chemo) therapies in patients harboring these specific mutations. Despite this advancement, the use of these drugs has only been associated with a 1.5 year average increase in survival [5].

This is because no drug has thus far been found that is immune to the development of resistance. Due to cancer’s genetic instability and composition of a heterogeneous population of cells, new mutations are able to arise in response to treatments allowing cells containing them to continue to grow and expand [6]. One specific cell population in the heterogeneous tumor has increasingly come into focus as the cells responsible for drug resistance and tumor recurrence in patients following treatment; the cancer stem cells (CSC). CSCs possess unique properties derived from their genetic and epi-genetic make-up that allow them to both escape treatment and initiate tumors. There is a great unmet need to develop novel treatments specifically targeting CSCs in order to overcome drug resistance and prevent patient relapse.
2.2 **Definition and History of CSCs**

CSCs have been characterized, ultimately, as a type of cell with the ability to initiate the growth of a tumor. They were originally discovered in acute myeloid leukemia (AML) patients via this tumor initiation property in 1994 by Lapidot et al. and tumor initiation ability remains the “gold standard” for verification of putative CSC populations to the present day. They are further defined as having two key attributes; self-renewal, and differentiation (Figure 1). This means that they can both divide symmetrically to produce more CSCs as well as asymmetrically to produce the non-stem cells that make up the bulk of the tumor. As shown in Figure 1, this mechanism allows CSCs that survive treatment to re-populate tumors with differentiated non-CSCs.

![Figure 1. Role of CSC in the Tumor Microenvironment](image)

*Model of the mechanism by which CSC can mediate the re-growth of tumors following treatment through their self-renewal and differentiation properties.*
These properties were also identified by the initial studies in AML as only cells possessing certain markers (CD34+ CD38-) were found to be able to self-renew and thereby establish cancer in mice [7]. When these populations were later isolated from the mice and examined they were found to contain a mixture of CD34+CD38- and CD34+ CD38+ revealing the CSC’s ability to differentiate. These studies also highlighted the rarity of CSCs within the total population of cancer cells. They were found to represent about 1 in every 250,000 cells in patient samples. Although this description of CSCs in AML was groundbreaking, it was not altogether unbelievable since AML is a cancer arising in hematopoietic stem cells, so it follows that some cellular mechanisms for self-renewal and pluripotency could be dysregulated or re-activated in this cancer.

On the other hand, the case for the role of CSCs in solid tumors, especially those of epithelial origin was not so easily made. First, simply finding stem-like cells within tumors does not equate to finding CSCs since it is known that mesenchymal stem cells (MSCs) hone to tumors and play an important role in the microenvironment by supporting tumor growth [8, 9]. Tumor infiltrating MSCs were implicated in supporting the growth of CSCs as well [10, 11]. Unlike CSCs, MSCs do not have the ability to initiate tumors on their own. Secondly, the lack of any universal marker for CSCs caused much debate among researchers as to whether specific cells isolated by any one group were truly CSCs. This challenge was compounded by the fact that many different models were used which sometimes gave inconsistent results. Doubt was even cast on the “gold standard” of CSC verification, tumor initiation in mouse models, due to the fact that experimental factors such as enzymatic digestion protocols, growth factor
stimulation, and differences in genetic background of mice strains made results difficult to replicate between groups [12, 13].

Over the years, however, evidence has continued to accumulate for the tumor-initiating role of cancer cells with stem like properties within many solid tumors. One key piece of evidence is the use of lineage tracing experiments to overcome the limitations inherent to xenograft tumor initiation models, thus revealing how single cells possessing a functional marker such as leucine-rich repeat-containing G-protein coupled receptor (Lgr5) can expand to create a large proportion of the resulting tumor [14]. Genetic lineage tracing also allows for the observation of expansion of cell populations in situ without disturbance by linking recombinase-reporter constructs to a marker gene of interest such that all daughter cells arising from a single parent will express the same reporter. While xenograft tumor initiation experiments had previously revealed that CSCs were more likely to be found in sub-populations expressing certain markers, lineage tracing has allowed researchers to pinpoint specific CSC cells and observe their clonal expansion as they grow within tumors [14-16].

These experiments have added convincing support for the role CSCs play in the growth of solid tumors, but they are also currently revealing new insights into CSC plasticity and the dynamics of cell populations within tumors. They have found that clonal expansion of cancer cells varies depending on cancer type, stage, and treatment. Showing that in some cases dominant clones arise that out compete others to eventually compose the bulk of the tumor in a steep hierarchy where small numbers of dividing CSCs feeds the bulk differentiated tumor (Figure 2). In other cases (such as melanoma) many cells within the tumor have the self-renewing property needed to
proliferate resulting in a tumor with less hierarchy and much more genetic diversity (Figure 2) [14, 17, 18]. Evidence for CSC plasticity arises from studies of targeted destruction of cells with CSC markers (Lgr5) and the observation that cells previously thought to be differentiated were able to re-gain expression of CSC markers and the ability to self-renew [15, 19, 20]. Wnt/β-catenin signaling has been implicated in this process but much work remains to be done [21].

Figure 2. Proliferation and Differentiation of CSCs Create a Hierarchy of Lineages Within Tumors
Application of the CSC model reveals differences in hierarchical organization of tumor cell populations which can vary depending on cancer type.
2.3 CSCs Clinical Relevance

CSCs have shown to be resistant to drug treatments and cause patients to relapse by initiating the growth of new tumors, so an understanding of CSC development and expansion is critical to understanding drug resistance and metastasis. For these reasons, treatments targeting CSCs remain a highly sought after yet elusive goal. CSC populations have thus far been characterized in patient tumors of nearly all cancer types, each with their own sets of identifying biomarkers. These include lung, breast, liver, colon, prostate, bladder, glioma, leukemia, and melanoma among others [22-24]. Examples of markers that have been used to identify CSCs directly in patient samples include aldehyde dehydrogenase (ALDH)+ and CD133+ cells in NSCLC, CD44+/24- and ALDH+ cells in breast cancer, CD34+/38- in AML, and CD26+ and Lgr5+ cells in colon cancer [20, 25-29].

In the current clinical environment where treatments to target CSCs are still in development, these CSC markers have been successfully used as prognostic markers to evaluate a patient’s disease and their response to treatment [26, 29, 30]. The success of these molecules as prognostic markers further highlights the need for CSC targeted therapies. Clinicians have found, independent of the underlying CSC hypothesis, that patient’s whose tumors contain more CSC markers tend to have poor outcomes and when CSC markers are lower patients have better outcomes.

2.4 Properties and Identification of CSCs

In recent years, evidence has been steadily accumulating in support of the roles CSCs play in tumor growth, metastasis, epithelial to mesenchymal transition (EMT), and
drug resistance [23, 31-35]. CSCs have been found to be resistant to drug treatment either by reduced metabolic activity/entry into a non-dividing quiescent state which confers resistance to genotoxic chemotherapy or by increased activity of drug efflux and breakdown pathways [34, 36, 37]. In addition, CSCs have frequently been associated with EMT that occurs as a precursor to metastasis leading them to also be found as circulating tumor cells [26, 31, 36, 38, 39]. A common marker for CSCs with a role in metastasis is C-X-C chemokine receptor type 4 (CXCR4). Its expression allows cells to migrate toward its ligand, CXCL-12, which is produced by bone marrow stromal cells among others and is commonly associated with metastasis to bone [40-42].

A universal biomarker for CSCs has yet to be discovered, however several cell surface markers, as well as functional assays, have been accepted as biomarkers for CSCs both *in vitro* and *in vivo*. For example, CD133 expression and the activity of the drug efflux pump ABCG2, as determined by side population assay, have become popular CSC markers in lung cancer, CD44+/CD24- cells are generally accepted as CSCs in breast cancer [28, 31, 43-46]. In addition, enrichment of CSCs can be shown by an increase in the expression of classical stem cell related transcription factors (Sox2, Oct4, and Nanog) conferring self-renewal and pluripotency [40, 47-49]. Activity of the ALDH enzyme, which is also active in normal adult stem cells, has been used to identify CSCs in multiple cancer types including breast, lung, and melanoma [25, 50-53]. Despite the identification of these markers, the study of CSCs has been limited by their rare occurrence in tumors, which can be as little as 0.05-1.5% [54, 55].

Because no universal marker exists, the “gold standard” for identification of CSC enrichment currently remains their ability to initiate xenograft tumors in mice at very low
dilutions [23, 56]. Even this assay is not without its limitations, as tumor initiation ability has been shown to vary with the genetic background of the mouse used, in addition to the fact that most models using human tumor explants lack the immune component of the tumor stroma. Nevertheless, *in vivo* tumor initiation is ultimately able to identify the presence of CSCs in a given cell population through their ability to form a tumor where non-CSCs are not able to do so.

### 2.5 Drugs Targeting CSCs

Many strategies have been used to try and target CSCs. Some such examples are targeting developmental pathways (Wnt, Notch, hedgehog) that are re-activated in CSCs with novel chemical inhibitors, targeting the oxidative/glycolytic metabolism that CSCs use for energy generation, and targeting stromal components that stimulate CSC growth such as cancer associated fibroblasts’ release of TGFβ or tumor associated macrophages [23, 57-59]. However, none of these strategies have been translated into a successful clinical treatment, although they are still being actively pursued.

Despite numerous clinical trials, many of the novel agents targeting developmental pathways fail as drugs because they are toxic to the normal adult stem cells that rely on these pathways and that are required for basic bodily functions [60]. In addition, because these pathways often contain complex regulatory mechanisms and redundancies it is unlikely that any single agent will ultimately be effective in targeting CSCs. Cancer cells will continue to develop new mutations and gene expression patterns to route around the particular signaling molecule that is inhibited. Therefore, interest is shifting towards combination therapies.
Through their ability to target multiple pathways or multiple elements of the same pathway simultaneously, combination therapies may prevent any single new mutation that arises from conferring drug resistance. This leads to the present challenge of finding drug combinations that are effective in targeting CSCs without being overly toxic to healthy cells. While work remains ongoing, the development of improved *in vitro* and *in vivo* models (such as the 3D scaffold presented herein) to facilitate drug screening will be vital in the search for effective combination therapies.

2.6 Concluding Remarks

In conclusion, sufficient evidence has accumulated not only for the existence of CSCs in solid tumors but also their critical roles in tumor growth, drug resistance, and metastasis. Therefore, the development of novel therapies to target CSCs holds great promise to improve patient outcomes. To efficiently study CSCs, novel *in vitro* models are desperately needed to overcome the fact that CSCs make up a very small fraction of cancer cells. Furthermore, the molecular mechanisms driving their expansion remain incompletely understood. We hypothesize that a fibrous scaffold 3D culture environment will promote CSC expansion through enhanced cell-cell and cell-matrix interactions creating a more representative model of CSC growth by eliminating artificially introduced reprogramming factors and growth factors. We further hypothesize that drug treatments targeting CSCs can be identified using this *in vitro* model. They will also be more effective in reducing tumor burden than traditional chemotherapies and targeted therapies through elimination of the self-renewing cell population that drives tumor growth and the development of drug resistance.
CHAPTER 3
GENERAL METHODS

This section presents the general molecular biology techniques used during the study.

In vitro Studies:

3.1 Assay for Reactive Oxygen Species

To assay for the production of reactive oxygen species (ROS) Cells were plated in monolayer culture 24hr prior to treatment. LLC1 cells were treated with 0.4nM Actinomycin D, 1µM Telmisartan, or the combination for 48hr prior to staining. The dye CM-H2DCFDA was used according to manufacturer’s instructions. Briefly, cells were stained in PBS containing 5µM CM-H2DCFDA at 37°C for 20 minutes. Following staining cells were washed in PBS. Staining was assayed immediately using fluorescence microscopy with a GFP filter. Bright field images were also acquired for reference. Staining was also assayed by flow cytometry (BD FACS Canto/Diva).

3.2 Beta Catenin Activity Assay

Active β-catenin was assayed in monolayer and 3D cultures using a Cignal TCF/Luciferase reporter system (Qiagen). Cells were transfected with the control or reporter plasmid using Lipofectamine 3000 reagent on day 3 of 3D culture (Thermo Scientific) and treated with drugs at stated concentrations 48 hours after transfection. Luciferase
activity (firefly reporter and renilla transfection normalization control) was assayed using the Dual Glo luciferase assay (Promega) in a BioTek Synergy H4 plate reader after 48 hours of treatment.

### 3.3 Cell culture

Polymeric nanofiber scaffold was prepared as previously described [61]. Briefly, a chloroform/dichloromethane solution containing mPEG-PLA and PLGA polymers was electrospun using a spraybase instrument at 20kV at a flow rate of 0.5mL per hour to create the sheet of randomly aligned fibers. Scaffolds were sterilized in ethanol, washed three times with PBS, additionally sterilized under UV light for 45 minutes, and then washed in cell culture media. Cells were seeded onto scaffolds in appropriate culture media (DMEM or RPMI containing 10%FBS and 1% penicillin/streptomycin) in standard cell culture well plates. Cell lines were obtained from the American type culture collection (ATCC). Drug tolerant H1975 were created by continuous exposure to Lapatinib in culture at a concentration of 7.5µM for 20 days. IC50 values for Lapatinib of parental H1975 and drug tolerant H1975 were compared to verify drug tolerance using the CellTiter-Glo. Cells were cultured in a humidified incubator at 37°C in a 5% CO₂ atmosphere (Thermo Fisher). 3D Tumoroid formation was assessed using fluorescent microscopy (Olympus BX51) after nuclear staining with Nuc Blue dye (Thermo Scientific).
3.4 CRISPR/Cas9

Plasmids containing CRRISPR/Cas9 constructs as well as scrambled control were purchased from Genecoepia. Cells were transfected using Lipofectamine 3000 according to manufacturer’s instructions. Transfected cells were selected using 5µg/mL puromycin and plated at a concentration of 100 cells/mL to obtain colonies composed of individual clones. These colonies were expanded in culture for downstream experiments.

3.5 CXCR4 Promotor Activity Assay

LLC1 cells were transfected using lipofectamine with a CXCR4 promoter reporter plasmid (MPRM14968-PG04, Genecopia) (1391 bp) containing a luciferase gene as reporter for CXCR4 activity and a secreted alkaline phosphatase gene as a control for transfection. Luciferase activity was assayed in the presence or absence of NPRA inhibitor using a luciferase based promotor reporter assay (Gluc-ON). Results were normalized for transfection efficiency using secreted alkaline phosphatase control and the Secrete-Pair assay kit (Genecoepia) Cells were stimulated with 1µg/mL lipopolysaccharide (LPS) and treated with 40µM Anantin.

3.6 Flow cytometry

All flow cytometry experiments were performed using a Becton Dickenson (BD) FACS Canto II system at the University of South Florida COM Fred Wright Jr Flow Cytometry Core. Cell sorting was performed on a BD FACS Aria system at the Flow Cytometry
Core at the H. Lee Moffitt Cancer Center & Research Institute. ALDH assays were performed using the ALDEFlour kit (Stem Cell Technologies) according to manufactures instructions. Controls Treated with Diethylaminobenzaldehyde (DAEB) were used to distinguish ALDH positive cells from background fluorescence. Antibody staining to determine CD44 and CD24 expression was performed using FITC or APC-Cy7 conjugated anti-CD44 and Alexa 647 conjugated anti-CD24 from BD. Cells were stained on ice for 25 minutes in FACS buffer consisting of PBS containing 5% FBS and 1mM EDTA. Dead cells were identified and excluded using 4′,6-diamidino-2-phenylindole (DAPI) staining. Analysis was done in BD FACS Diva software.

3.7 IC\textsubscript{50} Assay

IC\textsubscript{50} of drug treatments in 3D or monolayer culture was determined by Cell Titer Glo Assay (Promega) Culture media was changed to media containing drug dilutions on day 4 of culture and viability assay was performed after 48 hours on day 6 of culture. Cell Titer Glo reagent was added and incubated according to manufacturers’ instructions and luminescence was measured in a white well-plate in a Bio-Tek Synergy H4 plate reader. Actinomycin D was obtained from Acros Organics and Telmisartan was obtained from Selleckchem. Dose response curves were plotted and IC\textsubscript{50} values were estimated using Graph Pad Prism software.
3.8 Immunofluorescence Staining

Immunocytochemistry for Nos2 (Cell Signaling #D6B6S) was performed on LLC1 tumoroids cultured for 6 days on scaffold. Tumoroids were fixed and permeabilized on scaffold prior to staining.

3.9 Magnetic Cell Separation

CD24 expressing cells were depleted from A549 cell cultures using magnetic cell separation columns and CD24 antibody conjugated magnetic beads (Miltenyi Biotech) according to manufactures’ instructions. Depletion was verified using flow cytometry as stated above.

3.10 qPCR Array

Gene expression analysis was performed on LLC1 3D cultures using the cancer stem cell RT² PCR profiler array (Qiagen). RNA was isolated as stated above and processed according to manufactures” instructions. Array plates were read in a Bio Rad CFX-384 thermocycler and analysis was performed using the Qiagen Gene Globe Data Analysis Center web application.

3.11 Quantitative Reverse Transcriptase PCR

Total RNA was isolated from tissues or cell pellets using RNeasy columns (Qiagen) cDNA synthesis was performed using the Maxima cDNA synthesis kit (Thermo Scientific) according to manufacturer protocol. Real time analysis was performed using
a SYBR Green assay (Genecoepia) in a Bio Rad CFX-384 thermocycler using primers obtained from Integrated DNA Technologies. (See Table 1 for primer sequences). Data analysis was performed in Bio Rad CFX Maestro software with a significance threshold of \( p \leq 0.05 \).

3.12 Transcriptome Profiling Using RNA Quantification Sequencing

Transcriptome Profiling by RNA-Seq was performed by our collaborator Dr. Manoj Bhasin. RNA derived from LLC1 tumors was subjected to next-generation sequencing (NGS) to generate deep coverage RNASeq data. For each treatment group, sequencing was performed on 2 biological replicates. Sequencing libraries of Poly A selected mRNA were generated from the double-stranded cDNA using the Illumina TruSeq kit according to the manufacturer's protocol. Library quality control was checked using the Agilent DNA High Sensitivity Chip and qRT-PCR. High quality libraries were sequenced on an Illumina HiSeq 2000. To achieve comprehensive coverage for each sample, we generated ~20-25 million paired end reads for each sample.

RNASEQ data analysis:

The Raw sequencing data was processed to remove any adaptor, PCR primers and low quality transcripts using FASTQC and fastx/cutadapt. These provide a very comprehensive estimate of sample quality on the basis of read quality, read length, GC content, uncalled based, ratio of bases called, sequence duplication, adaptor and PCR primer contamination. These high quality, clean reads were aligned against human genome using hisat2. We used GRch38 human genome assembly as reference
genome for alignment. Gene expression measurement was performed from aligned
reads by counting the unique reads using featureCounts algorithm. The read count
based gene expression data was normalized on the basis of library complexity and
gene variation. The normalized count data was compared among groups in paired
manner using limma models to identify differentially expressed genes. The differentially
expressed genes were identified on the basis of raw P value and fold change. Genes
were considered significantly differentially expressed if the multiple text corrected p-
value was <.05 and absolute fold change >2.

**Functions, Pathway and regulatory network analysis**

Ingenuity Pathway Analysis (IPA, Qiagen) was used to identify the pathways that are
significantly affected by significantly differentially expressed genes. The knowledge
base of this software consists of functions, pathways and network models derived by
systematically exploring the peer reviewed scientific literature. A detailed description of
IPA analysis is available at the Ingenuity Systems’ web site (http://www.ingenuity.com).
It calculates a p-value for each pathway according to the fit of users’ data to the IPA
database using one-tailed Fisher exact test. The pathways with p-values <0.05 were
considered significantly affected. Further systems biology analysis was performed using
upstream regulators enrichment approach to identify upstream transcriptional regulators
that can explain observed transcriptome changes. Regulatory analysis help in
identifying significantly activated or inhibited transcriptional regulators on the basis of
upregulation or downregulation of its target genes. The significance of transcriptional
regulators activation/inhibition was determined using one-tailed Fisher’s Exact test. The
regulators with a P value <.01 and absolute Z-score ≥ 2 were considered statistically significant.

3.13 Sphere Formation Assay

Sphere efficiency was determined using sphere formation assays. Sphere media was prepared as previously described [62]. Cells were plated at a density of 2 cells per µL in sphere media in low attachment plates (Corning). Drugs were added 24 hours after cell plating and spheres were imaged using bright field microscopy after 6 days of culture. Sphere size and diameter was measured using ImageJ software statistical analysis was performed in Graph Pad Prism software. Sphere efficiency is represented as the percentage of cells seeded that were able to proliferate under low attachment conditions and defined as the number of spheres counted at endpoint divided by the number of cells seeded times 100.

3.14 Western immunoassay

Immunoassays for relative protein abundance were performed using Wes according to manufacture instructions (Protein Simple) Total protein was isolated using RIPA buffer and protein concentration was determined by Bradford assay. Caspase3 (Cell Signaling #9662p) PARP (Cell Signaling #9542) β-actin (Sigma #A2228).
3.15 Wnt Pathway Phospho-Antibody Array

LLC1 subcutaneous flank tumors were treated and collected as described above. Tumors from each treatment group were snap frozen on dry ice and stored at -80°C. Protein was isolated using an antibody array assay kit (Full Moon Biosystems) and loaded onto Wnt phospho explorer array slides (Full Moon Biosystems) per manufactures’ instructions.

In Vivo Studies:

3.16 Animal Experiments

C57/Bl6 mice were purchased from Envigo and NSG immunocompromised mice (NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ) were purchased from Jackson Laboratory. Subcutaneous tumors were grown in the flanks of mice by injecting one million LLC1 monolayer cells, 100,000 LLC1 3D cultured cells, or 5 million A549 cells. Tumors were allowed to grow and treatment was started when they became palpable (2-3mm diameter). Drugs were injected intratumorally once every 3 days at the concentrations treated with 50µg/kg Actinomycin D (AD), 1mg/kg telmisartan (TS), or combination. Drug solutions were made in PBS with 1%DMSO and this solution was used as vehicle control. Tumors were collected when controls reached 10mm in diameter. To obtain single cell suspensions of tumors the tissue was digested using a mouse tumor digestion kit and Gentle MACS instrument (Miltenyi Biotech) according to manufacturer instructions. Animals were housed in the University of South Florida comparative
medicine facility at the Morsani College of Medicine and all protocols were reviewed and approved by the USF institutional animal care and use committee.

**General Statistics:**

**3.17 Statistics**

Experiments have been repeated at least twice. Statistical significance for each experiment was determined using Analysis of variance (ANOVA) and the Tukey post hoc test, p<0.05. Calculations were performed and graphs plotted using Prism 6.0 software (Graphpad Software, San Diego, CA, USA). Graphs of results show the mean and error bars depict the average, +/- standard deviation.
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CHAPTER 4
Culturing Cancer Cells on the 3D Scaffold Increases Their Stemness

4.1 Introduction
Metastatic and drug resistant cancers are responsible for the vast majority of cancer-associated morbidity and mortality[24, 63]. Therefore, novel strategies to target these aggressive forms of the disease are desperately needed. CSCs, which comprise a minority (<1%) of the population of cells within tumors, possess self-renewal and multi lineage differentiation potential and are thought to be responsible for drug resistance in cancers. CSCs can be identified using several methods including the use of surface markers, expression of transcription factors, and functional assays. However, the populations identified by these methods can vary greatly among the types of experimental model used and may or may not represent distinct cell phenotypes[64-70]. It has been suggested that the tumor microenvironment (TME) plays a pivotal role in CSC proliferation, differentiation, and contributes to development of drug resistance. However, the precise cellular and molecular mechanisms responsible remain to be elucidated and the development of an adequate in vitro model for such analyses remains a critical unmet need. To this end, the broad long-term goal of this project is to develop an in vitro model replicating in vivo tumor matrix and cell-cell interaction that promotes proliferation of the CSC population. Then to use this model first, to investigate the molecular mechanisms responsible for the expansion of drug resistant and
metastatic CSCs, and secondly to identify a novel therapeutic combination treatment to target CSC.

This work is based on past developments in our lab. A 3D polymeric nanofiber scaffold as a substrate for cell growth was developed, which provides structural support to the cells partially replicating the function of extracellular matrix [61]. The scaffold developed in our lab is an electrospun mat of fibers consisting of a mixture of two polymers; methoxy-polyethylene-glycol(poly lactic acid) copolymer and polylactic-co-glycolic-acid. Previous studies from our Lab indicate that cells cultured on the scaffold exhibit some altered phenotype compared to cells grown as a monolayer resulting in increased resistance to anti-cancer drugs as well as increased expression of EMT markers; two important traits of CSCs [61]. This scaffold functions to increase cell-cell and cell-matrix interactions compared to 2D culture, thus resulting in the growth of 3D tumor organoids referred to as “tumoroids”. The availability of this system has led to the following hypotheses: (i) When cells are cultured on the scaffold their expression of genes and proteins are expected to be altered to a profile more similar to that found within primary tumors where CSCs develop. Profiling of the molecular changes that take place when cells are cultured on scaffold could lead to insight into the mechanism by which CSCs develop in vivo and identify new chemotherapy targets for CSCs. (ii) This 3D culture system may serve as a platform to enrich rare CSC population for downstream mechanistic studies of the factors influencing CSC development and for drug screening. Therefore, this 3D tumoroid model will be used to study and further define the mechanisms by which CSC proliferate and their population expands. We are proposing to expand the CSC population in culture and identify the molecular
mechanisms responsible for this expansion. To accomplish this, we will determine changes in cell phenotype that are caused by culture on 3D scaffold and whether these changes represent an enrichment of CSCs. Mouse and human lung cancer cell lines will be tested for changes in multiple markers and functional assays, including expression of transcription factors and stem cell surface markers. We will determine whether these changes represent an enrichment of CSCs by testing for known CSC cellular functions and confirm these results in a mouse model where putative CSCs will be tested for enhanced tumor initiation.

3D cultures provide significant advantages over 2D to study CSCs. The significance of the role of CSC populations in disease progression is becoming increasingly recognized by researchers. However, the characteristics of CSCs remain poorly defined [11-14]. CSCs identified by different methods have been found to behave in different ways and conflicting reports have been published claiming that the various identification methods do or do not identify a common population [1, 4, 15]. Many of these studies have been carried out in a 2D culture environment or in suspension culture where no extracellular matrix or other stromal environment is present [15-18]. Cells grown in these types of culture environments will adapt to the foreign conditions by altering their gene expression, therefore becoming less representative of in vivo tumors and less relevant models of disease [19, 20]. The fact that CSCs can make up as little as a fraction of one percent of the total cells grown in 2D cultures means they are difficult to isolate and even more difficult to maintain in culture once isolated. In order to more effectively study CSC populations, a culture environment that supports their growth and development is needed. To overcome the
fact that CSCs are such a small percentage of the tumor population, methods have been developed to expand CSCs in culture so they can be studied without interference from the differentiated cells that make up the bulk of tumors. These include artificial manipulations such as transforming cells with classical stem cell transcription factors (Sox2, Oct-4, c-Myc) or by exposing them to growth factors known to induce EMT (Wnt5a, TGFβ), as well as by changes to the structure of the culture environment such as hanging drop, suspension culture, or growth on hydrogels with or without extracellular matrix components [39, 71-74].

Of these, the most commonly used is suspension culture where anoikis resistant cells are grown into floating “tumorspheres” in the presence of the growth factors epidermal growth factor (EGF) and fibroblast growth factor (FGF) [62, 75, 76]. This has been shown to increase CSC marker expression and traits in functional assays. The ability for cells to survive in the absence of attachment is an indicator of the latent CSC potential within a given parental population. However, this technique has disadvantages, including the low CSC number obtained and re-differentiation once cells are removed from the growth factor containing tumorsphere media [22].

Synthetic culture substrates such as microspheres or fibers made from poly-caprolactone or poly-lactic co-glycolic acid have been used although much less frequently [61, 74]. These have the advantages of large surface area for cell growth as well as porosity to oxygen and nutrients, which have shown (by our lab as well as other groups) the ability to induce EMT and increase expression of stem cell transcription factors. Finally, CSCs have been directly isolated from in vivo tumors (either patient or mouse model) through methods such as fluorescent or magnetic cell separation based
on CSC marker molecules, but these methods have a high cost and result in low numbers of CSCs obtained [22]. It is these advantages and published results that have motivated the present work developing, studying, and validating a synthetic 3D scaffold model for CSC expansion and CSC targeted drug screening.

The majority of work in the 3D cell culture field is currently focused on hydrogels or matrigel [11-15]. While these are important and useful advances, this type of material does have some drawbacks that we aim to overcome through the development of a fibrous scaffold environment. Gels are relatively permeable to solutes, but fibrous scaffolds are inherently more permeable allowing for better diffusion of nutrients and growth factors. Many gels have components of biological origin which can induce batch variation and create a complex environment that is not strictly defined [16]. Gels are also uniform in structure, whereas fibrous scaffolds can be designed with different pore sizes and fiber diameters to create micro structures for cells to interact with. The contribution of a 3D fibrous scaffold culture environment is significant because it will provide an in vitro platform to study CSC origin, development, and cell-cell interactions. It will also reveal the mechanisms by which the structure of the extracellular environment can influence CSC proliferation.

CSCs have been identified in most types of cancer including breast, prostate, renal, colon, pancreas, and lung [77-80]. However, they have only been extensively studied in breast cancer models and even within that narrow field controversy still exists regarding the optimal method to identify the population and whether the diverse methods available identify a common population [64, 81]. The work presented here is innovative, because we aim to generate a novel 3D culture system for CSC enrichment
and use it to determine the factors and signals that contribute to CSC proliferation. This work will allow CSC populations isolated by different methods to be cultured and studied more easily, thus leading to better characterizations and refinement of in vitro cancer models, as well as identification of drug targets to limit CSC development in vivo.

In lung cancer, the five year survival rate is less than 10-20% for NSCLC and 5% for SCLC [82]. This is due to the high rates of metastasis and drug resistance [83, 84]. The CSC population in lung cancer, which is likely responsible for these traits, has not yet been definitively characterized. A 3D fibrous scaffold material has been chosen to study this population because of the advantages it offers over traditional monolayer culture and mouse models [85]. Because monolayer cultures lack the 3D structure of the ECM, cell behavior is altered and therefore results generated do not reliably translate to the clinic [86, 87]. Humanized mouse models do a better job of recapitulating in vivo human cell behavior but they are very expensive and can be more difficult to work with. For example, removing a single cell type from tumors or creating cell type specific knockouts is very time consuming and technically challenging [86, 88]. The scaffold culture model will be easier to manipulate in a controlled in vitro environment while still providing the 3D structural complexity to replicate the in vivo environment. When cells are cultured on the scaffold they grow as “tumoroids”. We have also found that cancer cells cultured on the scaffold exhibit enhanced resistance to multiple anti-cancer drugs and increased expression of some CSC markers [85]. However, the extent of these phenotypic changes is unknown.

We hypothesize that expansion of the CSC population plays a central role in drug resistance and metastasis. We further hypothesize that culture on the scaffold will lead
to an enrichment of CSCs with enhanced proliferative and metastatic properties. The enriched CSCs within cell lines will be identified by increased expression of CSC markers, as well as increased tumor initiation ability in vivo. These hypotheses will be tested using the 3D platform developed in our lab that has the potential to enrich the populations of metastatic and drug resistant CSCs in lung cancer cell lines. The first step towards identification of a true population of lung CSCs is to determine what molecular changes take place when cells are cultured on the scaffold. We aim to more fully characterize the phenotypic changes that occur when human lung cancer cells and biopsies of subcutaneous murine tumors are cultured on the scaffold. Lung cancer cell lines containing mutations in their epidermal growth factor receptor (H1975 and H460) and also KRAS (H460) will be used in addition to EGFR wild type cell lines human(A549) and mouse (Lewis lung carcinoma 1 (LLC1)) to represent a range of NSCLCs with differing growth characteristics and tendencies to develop drug resistance. Mutation status of the cell lines used is summarized in Table 2.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Species</th>
<th>Cancer</th>
<th>p53</th>
<th>EGFR</th>
<th>KRAS</th>
<th>DRUG RESISTANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC1</td>
<td>Mouse, C57 Bl/6</td>
<td>Lung adenocarcinoma</td>
<td>WT</td>
<td>OVEREXPRESS</td>
<td>MUTANT</td>
<td></td>
</tr>
<tr>
<td>A-549</td>
<td>Human</td>
<td>Lung adenocarcinoma</td>
<td>WT</td>
<td>WT</td>
<td>MUTANT</td>
<td></td>
</tr>
<tr>
<td>H1975</td>
<td>Human</td>
<td>Lung adenocarcinoma</td>
<td>MUTANT</td>
<td>MUTANT</td>
<td>WT</td>
<td>Gefitinib/Erlotinib</td>
</tr>
<tr>
<td>H460</td>
<td>Human</td>
<td>Lung carcinoma</td>
<td>WT</td>
<td>MUTANT</td>
<td>MUTANT</td>
<td>Gefitinib/Erlotinib</td>
</tr>
<tr>
<td>H1299</td>
<td>Human</td>
<td>Lung carcinoma</td>
<td>NOT-EXPRESSED</td>
<td>WT</td>
<td>WT</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Table of Cell Lines Used and Their Mutation Status in Key Tumor Promotor/suppressor Genes
It is expected that culture of lung cancer cells on scaffold will cause significant changes to cell morphology and phenotype compared to monolayer culture. The study of altered gene expression seen in scaffold culture is expected to help identify key signaling cues that support CSC proliferation. This will provide new insights into the mechanism by which CSCs develop *in vivo*, as well as a platform for future studies on isolated or enriched CSCs. Our lab is uniquely positioned to carry out this research because we have previously developed the 3D fibrous scaffold that we will use for cell culture. We also have expertise working with the required experimental protocols and tools, support from the USF flow cytometry core, and extensive experience working with mouse models of lung and prostate cancer. Upon completion of this project, we will achieve a better understanding of the similarities and differences in CSC composition.
and behavior in different cell lines, as well as tumor biopsies. We will gain new insight into the signals that influence CSC propagation, which may be useful in identification of drug targets. We will have created and validated a novel culture environment that promotes the growth of CSCs to be used in future studies.

4.2 Results

To establish the tumoroid model of cancer cell growth on our 3D scaffold material we began by ensuring that mouse and human lung cancer cell lines would form tumoroids when cultured on the scaffold. First, the properties of the 3D material (fiber diameter, porosity, uniformity of fibers) was confirmed to be consistent with batches used in previous work by scanning electron microscopy. (Figure 4A) Once the production of uniform material was confirmed, mouse (LLC1) and human (H1975, A549, H460, and H1299) cell lines were seeded onto the scaffold in cell culture media and imaged using fluorescent microscopy after 6 days of culture to assess tumoroid formation. (Figure 4B-F)

In figure 4B, LLC1 were stained with the cell membrane stain, Calcein AM (green), and in figure 4 C-F human cell lines were stained with the nuclear stain, NucBlue (blue). In all cell lines, 3D tumoroids in scaffold cultures were roughly 50-200µm in diameter on day 6 of culture. To determine whether cells cultured as 3D tumoroids acquired CSC properties we collected tumoroids from LLC1 and A549 cultures on day 6 and assayed their expression of CSC related genes compared to monolayer culture using quantitative real time PCR. (Figure 5) In LLC1 tumoroids we found the stemness transcription factors Sox2, Oct4, and Nanog to be elevated along with the aldehyde
dehydrogenase gene, aldh1a1. In A549 tumoroids we also found expression of Sox2 and Nanog to be elevated along with the CSC surface marker CD44, although only the increase in CD44 was statistically significant.

Figure 4. Lung Cancer Cell Lines Cultured on 3D Scaffold form Tumoroids

A. Scanning electron micrograph depicting fibrous structure of the 3D scaffold.  
B. LLC1 cells grown as tumoroids on the 3D scaffold for 6 days stained with the membrane stain calcein AM (green)  
C-F. Human NSCLC cell lines grown as tumoroids on the 3D scaffold for 6 days stained with the nuclear stain NucBlue.
In addition to cell line derived tumoroids we have previously described the utility of the 3D scaffold to culture biopsy derived tumoroids [61]. This method has the advantage of including stromal cells such as cancer associated fibroblasts and myeloid derived suppressor cells in the tumoroids, which is a more accurate representation of the *in vivo* tumor/stroma microenvironment. Using the mouse LLC1 cell line, which is syngeneic to the C57 BL/6 mouse strain, facilitates the collection of biopsies from subcutaneous tumors. Fine needle aspirate biopsies were collected from LLC1 tumors

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**Figure 5. Expression of CSC Marker Genes is Increased in Lung Tumoroid Culture**

LLC1 or A549 cells were cultured as monolayer or on scaffold for 6 days. RNA was isolated using affinity column (Qiagen), and cancer stem cell marker gene expression in monolayer vs scaffold cultures was assayed by qPCR. N=3 *p≤0.05.
when they reached 7mm in diameter. Cells obtained from LLC1 biopsies were then cultured on monolayer or scaffold for six days. These cultures were subsequently fixed, permeabilized, and immuno-stained for their expression of Oct4 or Sox2 transcription factors (Figure 6). In agreement with the qPCR data in figure 6, LLC1 biopsy tumoroids stained more intensely for both Oct4 and Sox2 than LLC1 biopsy monolayer cells.

![Expression of CSC Transcription Factors is Increased in LLC Biopsy Derived Tumoroids](image)

Figure 6. Expression of CSC Transcription Factors is Increased in LLC Biopsy Derived Tumoroids

Immunostaining LLC1 biopsies for stem cell transcription factors. Cells were cultured for 7 days either as a monolayer (A and C) or on 3D scaffold (B and D). Cells were stained red for Oct4 (A and B) and green for Sox2 (C and D). DAPI (blue) was used as a nuclear stain. Scale bar = 100μm

Due to the fact that we had observed increased CD44 expression in A549 tumoroids by qPCR and that the CD44+/24- population is known to possess CSC properties in some cancers, we next assayed both A549 biopsy tumoroids and H460 tumoroids for their expression of CD44 and CD24 by flow cytometry. A549 biopsies
were obtained by subcutaneous growth of tumors initiated using the A549 cell line in NOD SCID gamma immunocompromised mice. A549 form a much more solid tumor than LLC, so needle biopsies were not practical. So, whole A549 tumors were collected when they reached 7mm in diameter and digested as described in the methods to obtain single cell suspensions. CD44 and CD24 expression was compared on freshly digested tumor cells and tumor cells cultured as tumoroids for 6 days. (Figure 7) The freshly digested tumor had a CD44+/24- population of approximately 10%, with the majority of cells CD44-/24-. After tumoroid culture the CD44+/24- population increased to almost 30% accompanied by a large increase in overall CD44 and CD24 expression, with the majority of cells CD44+/24+ in addition to the CD44+/24- population. CD44+/24- analysis was subsequently performed on H460 tumoroids compared to H460 monolayer with similar results. H460 monolayer was found to contain

![Image of flow cytometry results]

**Figure 7.** *Expression of the CSC Marker (CD44+/24-) is Increased in A549 Biopsy Derived Tumoroids*  
CD44+/24- population as assayed by flow cytometry in cells collected directly from sub-cutaneous A549 tumors vs cells collected from A549 tumors and cultured as tumoroids for 6 days.
approximately 9% CD44+/24- cells, with the majority of cells being CD44+/24+ (Figure 8). Tumoroid culture increased the CD44+/24- population to 33% seemingly through a reduction in CD24 expression. (Figure 9)

Since we observed an increase in aldh1a1 expression in LLC1 tumoroids, we aimed to confirm this result through assays of ALDH activity in mouse and human tumoroids compared to monolayer culture. Figure 10A depicts ALDH activity in the H1975 cell line as measured by flow cytometry using the ALDEFluor kit (StemCell Technologies) with the ALDH inhibitor N,N-diethylaminobenzaldehyde (DEAB) provided as a negative control for background auto fluorescence. We observed a 5.2% increase in ALDH activity in H1975 tumoroids. We also assayed ALDH activity in LLC1 tumoroids and found a larger 34.5% increase compared to LLC1 monolayer (Figure 9B). We continued to culture the LLC1 tumoroids, dissociating and re-plating the cells on fresh scaffolds every 6 days for three generations. By the end of the 3rd generation ALDH
activity had increased such that nearly 90% of cells in the tumoroids had higher activity than LLC1 monolayer. (Figure 9B) This effect represents a statistically significant increase in ALDH activity. (Figure 9C)

![Figure 9](image)

**Figure 9. Aldehyde Dehydrogenase Activity is Increased by Tumoroid Culture**

**A.** The ALDH+ population was assayed by flow cytometry using the ALDEFluor kit in H1975 cells cultured as monolayer or tumoroids.

**B.** The ALDH+ population was assayed by flow cytometry using the ALDEFluor kit in LLC1 cells cultured as monolayer or tumoroids for multiple 6 day generations.

**C.** Percent increase in ALDH+ cells in LLC1 tumoroids vs LLC1 monolayer. N=3 *p≤0.05
CSC markers have been shown to be increased in tumoroid culture, so we aimed to confirm the development of CSC properties in tumoroids using the functional assays of sphere formation and tumor initiation. As discussed in chapter 2, these functional assays are important to ensure that the CSC markers used in this model are truly identifying cells that can self-renew and are able to form tumors. When LLC1 tumoroids were dissociated and tested for their ability to form spheres under low attachment conditions they were found to have an increased sphere forming ability (2.8%) compared to cells taken from LLC1 monolayer (2.3%). (Figure 10A-B) Not all cells within 1st generation tumoroids were ALDH+, so the ALDH+ cells were isolated from tumoroid culture using FACS. The sphere efficiency of the ALDH+ population was found to be more than double that of monolayer culture at 4.8%. (Figure 10A-B) The sphere growth properties were observed to be slightly different between groups as depicted by

Figure 10. **Sphere Formation Efficiency is Increased by Tumoroid Culture and ALDH Activity**

A. Sphere efficiency of LLC1 cells derived from monolayer (M), scaffold (S), or ALDH+ population obtained by FACS. Sphere efficiency = cells plated/ # of spheres observed. N=3 *p≤0.05

B. Box plot showing size distribution and sphere efficiency % of spheres from (A).
changes to the size distribution of spheres (Figure 10B), with tumoroid cells growing a large number of small spheres while monolayer and ALDH+ cells grew a larger range of sphere sizes. This is most likely an indication of differences in the ability to proliferate but the specific mechanism causing this requires further investigation.

To investigate the tumor initiation ability of the ALDH+ LLC1 and CD44+/24- A549 populations, these populations were isolated from tumoroids by FACS or MACS, respectively and used to initiate subcutaneous flank tumors in C57 BL/6 or NOD SCIID gamma mice. The LLC1 ALDH+ were able to initiate tumors much more efficiently than parental LLC1 tumoroid cells at a concentration of 10,000 cells per flank. (Figure 11A)

![Graph A](image)

**Figure 11. CSC Populations Enriched by Tumoroid Culture have Enhanced Tumor Initiation Ability**

**A.** Tumor initiation ability of LLC1 CSC population. LLC1 cells cultured on 3D scaffold vs isolated ALDH high population were injected subcutaneously using 10,000 cells per flank and the size of the resulting tumors was measured by caliper. N=2 p=0.1

**B.** Tumor initiation ability of A549. Parental cells vs MACS isolated CD44+/24- population were injected subcutaneously into the flanks of Nod/SCIID immunocompromised mice using the cell numbers indicated for each group. N=3 *p≤0.05
The A549 CD44+24- cells were also able to initiate tumors more efficiently than parental, as CD44+/24- tumors seeded with 25,000 cells grew at a faster rate than parental tumors seeded with 5x10^6 cells and eventually surpassed the parental in size. (Figure 11B)

Figure 12. **Expression of CSC Marker Genes Changes Over Time During Tumoroid Culture**

**A.** A qPCR array was used to identify changes in RNA expression for cancer stem cell related genes in first, second, and third generation LLC1 scaffold culture. Data was normalized to LLC1 monolayer and fold change values are presented as a table.

**B.** Scatter plot showing up and downregulated genes in LLC1 3rd generation vs LLC1 monolayer.
Since LLC1 tumoroids, and especially the ALDH+ population they contain, were confirmed to have the key CSC properties of enhanced sphere formation and tumor initiation, their gene expression profile was further characterized using a qPCR array containing primers for genes known to be important markers and drivers of the CSC.

**Figure 13.** Expression of CSC Marker Genes is Enhanced by Tumoroid Culture in Multiple Cell Lines

A. Human NSCLC cell lines A549, H1299, and H460 were cultured at 3D tumoroids for 6 days or as monolayer. Cell pellets were collected from each culture, RNA was isolated and CSC related gene expression was analyzed by qPCR. Data represents increase in gene expression in tumoroids as compared to monolayer. One way ANNOVA was used to calculate significance (Prism) N=3 *p≤0.05.

B. Nos2 Expression in the human cell lines H1299, A549, H460 and mouse LLC1 cultured on scaffold for 6 days normalized to monolayer.

C. IHC staining for Nos2 protein in 1st Gen LLC1 tumoroids fixed on day 6 of culture and stained with rabbit anti mouse Nos2 and Alexa 488 anti-rabbit secondary. (600X magnification) *p≤0.05
phenotype. In order to better understand the mechanism by which culture on the 3D scaffold is able to promote the growth of CSC, samples from 1\textsuperscript{st}, 2\textsuperscript{nd}, and 3\textsuperscript{rd} generation tumoroids were used to identify gene expression changes that occurred over time in stem cell related genes. Several genes were found to be up-regulated including Nos2, PLAT, and CD34. Many of these are involved in Wnt/\(\beta\)-catenin signaling, a pathway known to drive cell proliferation and regulate cell-cell adhesion in cancer. (Figure 12A) A scatter plot of overall gene expression changes occurring between LLC1 monolayer culture and 3rd generation scaffold is also provided. (Figure 12B)

Nos2 showed the greatest increase in scaffold culture and this result was replicated in the human adenocarcinoma cell lines A549, H1299, and H460 as well as in LLC1 when cultured on scaffold. (Figure 13A) Immunohistochemistry (IHC) for the Nos2 protein was used to confirm its expression as seen in the qPCR array. (Figure 13B) Nos2 (stained in green) is highly expressed in LLC1 tumoroids. Many of the other stemness genes shown to increase in LLC1 cultured on scaffold were also increased in human cell lines cultured on scaffold for 6 days, albeit at a somewhat lower fold change. (Figure 13C) Taken together, this data demonstrates that the effects of the scaffold culture are not cell line specific and can promote stem-ness in human cancer cells as well as mouse cells.

\textbf{4.3 Discussion}

We are not the first to demonstrate an increase in CSC markers through culture on a fibrous scaffold, as Feng \textit{et. al.} have also reported this phenomenon reporting increased expression of CSC markers Oct3/4, Sox2, and ALDH in breast cancer cell lines cultured
on a polycaprolactone scaffold. However, we have significantly advanced the field by performing an extensive characterization of the phenomenon as well as validating the CSC expansion observed on our novel scaffold material using multiple in vitro methods as well as in vivo tumor initiation [89]. We have demonstrated that our tumoroid platform can be used with both mouse and human cells with varying genetic background and that it is able to expand the expression of relevant CSC markers to each cell line (ALDH in LLC1 and CD44+/24- in A549). In addition to our own validations, other groups have studied the relevance of ALDH in LLC1 cells and CD44 in A549 cells which adds support to the claim that tumoroids grown on our scaffold are truly enriched in CSCs [40, 90-92].

We have successfully utilized the tumoroid platform to generate and maintain large numbers of CSCs separate from their bulk tumor cells enabling full scale experiments such as RNA and protein isolation from CSCs, in vivo tumor initiation, and drug screening. Through the study of gene expression changes occurring as CSCs are maintained within tumoroids, we have identified the Wnt/β-catenin pathway as a major driver not only in mouse LLC1 cells but also in multiple human NSCLC cell lines. This phenomenon will be explored in greater detail in the coming chapters.

Nevertheless, some aspects of CSC expansion in the tumoroid model remain to be explored. The full significance of the NO production increase we observed in tumoroids remains unclear. Because Nos2 is a Wnt target gene it provides some evidence that β-catenin activity is increased by scaffold culture. iNOS is known to promote drug resistance in lung cancer by activation of the canonical Wnt pathway through inhibition of DKK1 and to promote the growth of CSC in glioma via the Notch
pathway [93]16, 17). In this way, iNOS may be acting in a positive feedback loop to promote stemness in tumoroid cultures. The NO which is released by these CSCs may also play a role in increasing vascular permeability and inducing immune cell anergy, but this area requires further investigation.

The significance of the difference in expression patterns of individual CSC target genes seen in figures 13-14 may provide some insight into how CSCs arise in the presence of differing driver mutations. For example, even though Wnt/β-catenin is implicated in the process of CSC proliferation, different Wnt genes were observed to be active in each cell line with some such as the common Wnt responsive gene c-myc not observed to increase at all [94, 95]. This is most likely due to the context specificity of the Wnt pathway and its many segments (both canonical and non-canonical) that will be discussed in detail in chapter 7. Despite the status of c-myc as a reporter of canonical Wnt activation, alternative Wnt activation can actually cause a decrease in c-myc expression especially in the context of increased CD44 [96, 97]. Despite the present validation of the tumoroid model of CSC proliferation, the details of the molecular mechanism behind the increased CSC proliferation remain unclear. Due to the facts that the 3D scaffold provides greater surface area for cells to attach and cells cultured on the scaffold come together to form 3D tumor-spheres increasing their cell-cell interaction, it is logical that stimulation of cell adhesion molecules could be responsible for the expansion of CSCs that occurs in this model but more work must be done to determine the specific mechanisms involved.
CHAPTER 5

A Novel Mechanism Regulating CSC Expansion (Natriuretic Peptide Receptor A)

5.1 Introduction

Natriuretic peptide receptor A (NPRA) is the cell surface receptor for atrial natriuretic peptide (ANP). NPRA is found in many tissues including kidney, brain, ovaries, and endothelium [98]. NPRA functions as a guanylyl cyclase converting GTP into cGMP

Figure 14. The Functions of ANP/NPRA Signaling

ANP is produced by the heart in response to stress/high blood pressure. It is recognized by NPRA in the kidney leading to both natriuresis (excretion of sodium) and vasodilation through the renin angiotensin pathway. Because NPRA is a receptor guanylate cyclase it can also activate PKG in other tissues including in embryonic stem cells where it maintains pluripotency through an Oct4/Nanog dependent mechanism.
upon its binding to ANP which in turn can activate multiple downstream signaling pathways. ANP is a peptide hormone produced by the heart with diuretic and natriuretic properties [99]. This is the primary function of ANP/NPRA signaling; to decrease blood volume in response to cardiac stress as a mechanism to reduce blood pressure. However, ANP/NPRA signaling has also been shown to modulate inflammation and fibrosis in several animal models, as well as maintain self-renewal and pluripotency in embryonic stem cells [100-102]. (Figure 14) Natriuretic peptide receptors are found on immune cells including macrophages and dendritic cells, as well as MSCs [103]. NPRA specifically has been shown to reduce endothelial inflammation through its guanylyl cyclase activity [104]. Studies thus far indicate that the ANP-NPRA pathway plays a role in the healing process and may be an important contributor to the outcome of cardiovascular ischemia and myocardial infarction [105]. Studies from the Mohapatra lab have shown that a deficit in ANP-NPRA signaling reduces the SDF-1/CXCR4 mediated homing of MSCs to tumors, indicating that a link between the NPRA and CSC associated CXCR4 pathways exists. However, the exact mechanism of this link and the specific cell types involved remains unclear [106].

Studies from our laboratory have shown that a blockade of ANP-NPRA signaling can reduce CXCR4 expression in tumor microenvironments, as well as reduce angiogenesis in aortic segments. Co-culture with MSCs can partially recover these effects [106]. Initially, angiogenic potential of aortas derived from WT and NPRA-KO mice were compared using an endothelial sprout assay. Results showed that NPRA-KO aortic sections exhibit reduced sprouting (angiogenesis), which was restored when co-cultured with MSCs [106]. Reduced angiogenesis potential of NPRA-KO aortas co-
related with decreased VEGF production in the culture [106]. This indicates that the presence of MSCs can promote angiogenesis and may aid in healing at sites of vascular damage.

NPRA is overexpressed in many cancers but its role in tumor progression has not been extensively studied [107]. Because our previous studies suggest that NPRA is crucial for the growth of tumors these studies examining the specific role of NPRA in the tumor microenvironment with respect to drug resistance and CSC development will provide significant insight into how tumors progress to more aggressive forms and possibly identify novel targets to prevent this. Natriuretic peptide receptors are found on many cell types including ECs, MDSCs, and are overexpressed in many cancers [108, 109]. NPRA has been shown to play a role in tumor growth and angiogenesis [28]. However, the specific role of NPRA signaling in different cell types present in the tumor microenvironment is still unknown. Because CXCR4 and SDF-1 expression are reduced in NPRA KO tumors and recovered when NPRA WT mesenchymal stem cells are introduced it is likely that NPRA influences the migration and/or development of CAFs from MSC [106]. Previously published data from our group indicates that the NPRA pathway influences CXCR4 expression and is crucial to tumor formation in a knockout mouse model. Both CXCR4 and its ligand CXCL-12 were greatly reduced in NPRA KO tumors.

This data suggests that the NPRA signaling pathway is involved in tumor progression and may also be involved in CSC development. Therefore, the scaffold model will be used to study and further define the role that NPRA plays in the tumor-stroma interaction. We hypothesize that NPRA expression will be increased during CSC
expansion and a reduction in NPRA signaling achieved by gene knockout or small molecule antagonists will reduce both CXCR4 expression and CSC development in cells cultured on scaffold. We further hypothesize that NPRA disruption will have a direct effect in reducing the metastatic, proliferative, and stem like properties of cancer cells. We are proposing to expand the CSC population in culture as described in chapter 4 and identify the molecular mechanisms responsible for this expansion. We aim to elucidate the role of NPRA signaling in the development of CSCs and regulation of the expression of the CSC marker CXCR4. This will represent a candidate signaling pathway influencing the development of CSCs.

Upon completion of this aim, we will achieve a better understanding of the similarities and differences in CSC composition and behavior in different cell lines as well as tumor biopsies. We will gain new insight into the signals that influence CSC propagation, which may be useful in identification of drug targets. Furthermore, these studies will provide novel insight into the role of specific cells in the tumor microenvironment with respect to development of drug resistance and CSCs, as well as insight into how NPRA signaling in each cell type affects the tumor as a whole. Finally, we will have created and validated a novel culture environment that promotes the growth of CSCs to be used in future studies.

The proposed work is innovative, because we aim to determine the factors and signals that contribute to CSC proliferation. This work is also innovative because it will reveal for the first time the influence of the NPRA pathway on CSC development and specifically identify stromal conditions important to this process. This work will allow CSC populations isolated by different methods to be cultured and studied more easily.
leading to better characterizations and refinement of \textit{in vitro} cancer models, as well as identification of NPRA related drug targets to limit CSC development \textit{in vivo}.

5.2 Results

After establishing that CSCs are enriched in LLC1 tumoroids in chapter 4, we next examined weather the increase we saw in CSC markers was accompanied by an increase in NPRA expression. LLC1 tumoroids were stained for ALDH activity and co-stained with fluorophore conjugated antibodies for either NPRA or CXCR4. Samples were gated into ALDH high and ALDH low populations and back-gated to show the

![Figure 15. Increased ALDH Activity Coincides with Increased NPRA and CXCR4 Expression in Tumoroids](image)

- **A.** NPRA and CXCR4 expression in LLC1 cultured as monolayer.  
- **B.** NPRA and ALDH co-expression in LLC1 tumoroids.  
- **C.** CXCR4 and ALDH co-expression in LLC1 tumoroids.
distribution of NPRA or CXCR4 between them. LLC1 tumoroids with high ALDH activity were found to be 43% NPRA+. However, the NPRA+ cells (in purple) were fairly evenly distributed among the ALDH+ and the ALDH- cells. (Figure 15B) The same LLC1 tumoroids were found to contain 6% CXCR4+ cells (in orange), the majority of which co-stained for high ALDH activity (Figure 15C). Comparing between monolayer and tumoroids reveals that tumoroid culture caused a relatively small increase in NPRA cell surface expression in LLC1 but does cause a significant increase in CXCR4 expression. (Figure 15C)

A similar analysis was performed on H460 tumor spheres grown in low attachment conditions. Tumor sphere culture was observed to cause an increase in both NPRA

Figure 16. Increased CD44+/24- Population Coincides with Increased NPRA and CXCR4 Expression in Tumoroids
A. H460 cultured as monolayer and tumorspheres were assayed for NPRA expression and CD44+/24- population by flow cytometry. 
B. Expression of NPRA and CXCR4 genes were compared by qPCR in H460 monolayer and tumoroids. N=3 *p≤0.05
expression (from 6.4% to 40%) and the CD44+/24- population (16% to 26%). (Figure 16A) mRNA expression was also checked in tumor spheres vs monolayer. Both NPRA and CXCR4 expression was significantly increased in the tumorspheres.

NPRA was observed to increase in both the tumor sphere and tumoroid models, so NPRA levels were also analyzed in a model of lung cancer drug tolerance. H1975 cells were exposed to the epidermal growth factor receptor (EGFR) inhibitor, Lapatinib, at sub lethal concentrations to observe how they respond to treatment at the early stages of developing drug resistance. After 20 days of exposure to the drug it was determined that their IC\textsubscript{50} for Lapatinib was increased H1975-drug tolerant (H1975-DT) cells. (Figure 17A) Moreover, qPCR study revealed that the H1975-DT cells had

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**Figure 17. Drug Tolerance is Correlated with Increased NPRA Expression**

A. Increased IC\textsubscript{50} was confirmed in lapatinib tolerant H1975 cells by CellTiterGlo assay.
B. NPRA gene expression was assayed by qPCR in H1975 parental, and Lapatanib tolerant cells. N=3 *p≤0.05
C. NPRA receptor expression was assayed by flow cytometry in H1975 parental and Lapatanib tolerant cells.
significantly increased expression of NPRA. (Figure 17B) This increase was confirmed by flow cytometry, where H1975-DT were observed to have a 17% increase in NPRA on their cell surface. (Figure 17C)

NPRA expression was elevated in both models of CSCs and drug tolerance, so we wanted to test the effects of inhibiting NPRA signaling to determine whether the inhibition would have an effect on the expression of CSC markers. H1975 were cultured as tumoroids and their CXCR4 expression was assayed with or without treatment with the NPRA inhibitor, anantin. CXCR4 expression was observed to increase in H1975 tumoroids compared to monolayer and decrease in tumoroids treated with anantin.

(Figure 18A-B)
This experiment was repeated using LLC1 cells and it was observed that CXCR4, CD44+/24-, (Figure 18C) and ALDH+ (Figure 19A) markers are decreased in LLC1 tumoroids treated with anantin. After finding that NPRA inhibition was able to decrease cell surface CXCR4 in multiple cell lines, the effects of NPRA inhibition on the CXCR4 promoter were investigated using a luciferase based promoter reporter construct to determine if NPRA signaling regulates CXCR4 transcription. When LLC1 cells were stimulated with the inflammation inducing bacterial LPS their CXCR4
promotor was activated as expected, however when the cells were co-treated with anantin, CXCR4 promotor activity remained the same as untreated. (Figure 19B)

Due to several issues in the use of anantin to inhibit NPRA inherent to its cyclic polypeptide structure (high cost, short half-life, and high dose needed) alternate methods of NPRA inhibition were investigated to further study the effects of NPRA on CSC growth. Towards this end, a CRISPR Cas/9 gene knockout technique was used to create an NPRA knockout H1299 cell line. Following transfection with a commercially available all in one CRISPR plasmid targeting NPRA, individual cells were isolated to form clonally derived colonies. These colonies were tested for NPRA expression by flow cytometry.

Figure 20. Use of CRISPR/Cas9 to Create an NPRA Knockout Cell Line
A. H1299 cells were transfected with an all in one CRISPR/Cas9 plasmid construct containing guide RNA sequences for the NPRA gene. Following transfection, cells were cultured as individual colonies and colonies were selected and assayed for NPRA expression by flow cytometry. NPRA expression for colony #1 is plotted as both dot plot (A) and histogram (B) parental H1299 and secondary antibody only are provided as positive and negative controls.
cytometry to determine if the knockout was successful. One colony showed a significant reduction in NPRA from 68% to 14% (Figure 20A-B), so studies were continued with this colony to determine if its CSC properties were also reduced by NPRA KO.

A sphere assay was performed to test if NPRA KO has any effect on sphere efficiency and therefore self-renewal ability. Both parental H1299 and NPRA KO H1299 were able to form spheres in a similar distribution of sizes. (Figure 21A) However, the NPRA wild type H1299 formed ~twice as many spheres as the NPRA KO. This reduction in sphere forming ability in the NPRA KO H1299 indicated reduced stemness.

To validate this finding, the tumor initiation ability of NPRA KO H1299 was determined. NSG mice were injected subcutaneously with WT cells in the right flank and KO cells in the left flank and tumor growth was monitored by caliper measurement. Both WT and KO cells were compared in each mouse to control for individual variation between mice. Tumors from both WT and NPRA KO were observed to increase in size from day 30 post injection to day 50. However, in each mouse the NPRA KO tumor

<table>
<thead>
<tr>
<th>Sphere Diameter μM</th>
<th>Parental Ctrl</th>
<th>NPRA KO colony 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-50</td>
<td>8%</td>
<td>4.9%</td>
</tr>
<tr>
<td>60-80</td>
<td>6%</td>
<td>4.2%</td>
</tr>
<tr>
<td>80-100</td>
<td>5%</td>
<td>3.8%</td>
</tr>
<tr>
<td>100-120</td>
<td>4%</td>
<td>3.4%</td>
</tr>
<tr>
<td>120-140</td>
<td>3%</td>
<td>3.0%</td>
</tr>
<tr>
<td>140-160</td>
<td>2%</td>
<td>2.6%</td>
</tr>
<tr>
<td>160-180</td>
<td>1%</td>
<td>2.2%</td>
</tr>
<tr>
<td>180-200</td>
<td>0%</td>
<td>1.8%</td>
</tr>
</tbody>
</table>

Figure 21. **NPRA Knockout Reduced Sphere Formation Ability**
Sphere formation ability was assayed in NPRA-KO H1299 vs parental H1299 cells. The size distribution of spheres and the sphere efficiency values for each group are depicted. Sphere efficiency = cells plated/ # of spheres observed. N=3
grew more slowly and remained smaller in size compared to the NPRA WT tumor. (Figure 22A)

Due to the fact that we observed that NPRA KO in cancer cells did not prevent tumors from growing, we decided to investigate NPRA KO in the tumor stroma. To determine how NPRA KO in the non-cancerous cells of the tumor microenvironment would affect tumor growth we obtained commercially available NPRA KO mice of the C57 BL/6 background and used them to study the growth of LLC1 tumors in an environment lacking NPRA. Previous studies from our Lab have showed that LLC1 monolayer cells are not able to efficiently form tumors in NPRA KO mice, so we used LLC1 cells derived from tumor sphere culture to compare subcutaneous tumor growth in

![Figure 22. NPRA Knockout in Tumor or Stroma Reduces Tumor Growth](image)

A. NPRA KO H1299 cells were used to initiate tumors in NSG Mice and growth was monitored over 60 days (N=3). Mice were numbered 1-3 and each mouse received 3x10^6 WT cells in the right flank and 3x10^6 NPRA-KO cells in the left flank.

B. Subcutaneous flank tumors were grown in NPRA KO mice using LLC1 tumorspheres at the indicated cell numbers (x1,000) (N=2) *p≤0.05
NPRA WT and NPRA KO mice. Monitoring tumors initiated with decreasing numbers of cells revealed that tumors were able to grow much faster and to larger size in the WT mice when initiated with either 100,000 or 20,000 cells. (Figure 22B) No difference was observed in tumors initiated with 10,000 cells in the 30 day time period of the experiment. This is likely due to the much slower growth kinetics in tumors initiated with such a low cell number.

5.3 Discussion

While NPRA has been previously demonstrated to play a role in embryonic stem cell pluripotency and the migration of mesenchymal stromal cells, to our knowledge we have presented herein the first indication of NPRA’s role in maintaining CSC or drug resistant populations of cancer cells. Other groups are beginning to investigate the role of NPRA in cancer including studies showing that NPRA can increase invasion and migration in squamous cell carcinoma and promote gastric cancer development supporting our findings that it may play an important role in the progression of lung cancer [110-112].

We did not consistently see dramatic increases in NPRA expression as a result of tumoroid culture, and indeed have previously shown that NPRA is often overexpressed in cancer cell lines grown as monolayer, indicating that NPRA itself may not be a good marker for CSC identification [109]. Previously published work demonstrates that NPRA is overexpressed in many types of cancer including in the LLC1 cell line and in vivo tumors. Therefore, signaling through NPRA may be important in supporting survival and/or growth of these cancers. The results presented here
support this conclusion, as we have demonstrated that increases in NPRA expression are associated with increases in CSC markers (ALDH, CD44+/24-, and CXCR4) as well as functional properties of CSCs including drug resistance, self-renewal, and the ability for tumors to grow in vivo. Furthermore, we have demonstrated that by inhibiting NPRA signaling, either using anantin or NPRA KO, we were able to reduce the expression of both ALDH and CXCR4 in lung cancer cells and simultaneously reduce their self-renewal and tumor initiation.

While the role of NPRA in lung cancer CSCs requires further study, we have begun to reveal a possible regulatory mechanism by showing that inhibition of NPRA signaling can reduce the transcriptional activity of the CXCR4 promotor. This may lead to future therapeutic intervention as increased CXCR4 expression has been associated not only with CSC growth, but also metastasis of cancer cells and migration of stromal cells into tumors where they support tumor growth [41, 42, 106, 113].
CHAPTER 6
Actinomycin D and Telmisartan Combination Treatment Targets CSC and Reduces Tumor Burden

6.1 Introduction

As described in chapter 1, a major goal of this project is to utilize the 3D tumoroid CSC model to identify novel therapies to target and destroy CSC. Since we have established in chapter 4 that 3D tumoroids are indeed enriched in CSC we subsequently began testing anti-cancer drugs to determine whether they could block this expansion. We started by using a primary drug screen of the NCI diversity set of compounds in an established (monolayer) model of drug resistant cancer (MCF-7 Dox cell line) in order to identify lead compounds [114]. Subsequently, a secondary screen was performed using the NSCLC cell line H460 and Actinomycin D (AD) was identified as the compound able to reduce cell viability most potently in both screens (Table 3). In addition, we went on to determine that AD was also able to reduce Sox-2 expression in breast cancer tumoroids thereby reducing stemness [115]. Therefore, since we have identified in chapter 4 that ALDH expression is a valid marker for CSC in the LLC1 cell line, we hypothesized that treating LLC1 tumoroids with AD would result in a reduction in ALDH activity. We further hypothesized that if AD is successful in targeting CSC it would be effective in reducing the viability of not only LLC1 tumoroids but also
tumoroids generated from human NSCLC cell lines and that this anti-CSC effect would translate to in vivo mouse models of NSCLC as well.

Table 3. Drug Screening with the NCI Diversity Set in the H460 Cell Line

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin-D</td>
<td>0.03</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>0.0556</td>
</tr>
<tr>
<td>Plicamycin</td>
<td>0.2801</td>
</tr>
<tr>
<td>Daunorubicin HCL</td>
<td>0.3407</td>
</tr>
<tr>
<td>4'-Epipodophyllotoxin</td>
<td>0.3851</td>
</tr>
<tr>
<td>Doxorubicin HCL</td>
<td>0.4155</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>0.937</td>
</tr>
<tr>
<td>Mechlorethamine hydrochloride</td>
<td>1.127</td>
</tr>
<tr>
<td>Mitozantrone</td>
<td>1.44</td>
</tr>
<tr>
<td>Topotecan</td>
<td>2.19</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>2.525</td>
</tr>
<tr>
<td>Depsipeptide</td>
<td>4.727</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>6.913</td>
</tr>
<tr>
<td>4-DMD</td>
<td>7.58</td>
</tr>
<tr>
<td>Etoposide</td>
<td>8.81</td>
</tr>
<tr>
<td>Carfilzomib</td>
<td>10.06</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>10.38</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>11.34</td>
</tr>
<tr>
<td>Pazopanib</td>
<td>11.97</td>
</tr>
<tr>
<td>Sorafenib tosylate</td>
<td>12.78</td>
</tr>
<tr>
<td>PFZ341066</td>
<td>13.66</td>
</tr>
<tr>
<td>BIBW2992</td>
<td>15.9</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>17.1</td>
</tr>
</tbody>
</table>

The anti-cancer activity of AD has been known since the 1950’s when it was one of the first antibiotics discovered to be effective in the treatment of tumors [116, 117]. Its structure is a cyclic peptide, and it is produced by actinomyces soil bacteria where it was first discovered in the 1940’s [118]. Actinomycin D exhibits genotoxic anti-proliferative and anti-cancer effects by binding to DNA at GpC sites where it inhibits RNA polymerases from accessing the DNA thereby inhibiting transcription [119, 120]. It has also been shown to inhibit DNA topoisomerase activity resulting in increased stress.
on the DNA molecule and ultimately double-stranded breaks [120]. Through these mechanisms, AD has been shown to induce apoptosis in many cancer cell lines [115, 121-123]. Due to its potent activity, AD was approved by the FDA to treat Wilms tumor, rhabdomyosarcoma, and choriocarcinoma in 1964 under the trade name Cosmegen. Since its approval AD has been used as a chemotherapy in the clinic however its use and effectiveness have been limited by its dose-limiting side effects including immunosuppression, fatigue, ulcers, and diarrhea [124, 125].

In addition to its use as a primary chemotherapy, in recent years AD has been shown to be effective in cyclotherapy. The principle of cyclotherapy states that because many cancer therapies target rapidly dividing cells side effects of these therapies could be minimized if non-cancerous cells could selectively be made to stop dividing during treatment [126]. Towards this end, AD has been given in low doses to activate the P-53 DNA damage response in non-cancerous cells causing cell cycle arrest after which point they will not be harmed by high dose chemotherapies [123, 126, 127].

Because AD has been shown to be an effective cancer therapy both in the literature and by our preliminary screen, but its use is limited by side effects we considered combining AD with other drugs in an effort to reduce the dose required while maintaining its anti-cancer and anti-CSC effects. To achieve this goal, we have chosen the drug Telmisartan (TS), an angiotensin II receptor antagonist developed by Boehringer Ingelheim as an anti-hypertensive agent. It was selected for combination therapy with AD due to its well-known effect reducing collagen production in the tumor microenvironment thereby increasing the permeability of tumors and increasing drug penetration into them [128, 129]. However, TS is also known to have other effects in
models of cancer that make it an attractive candidate for combination therapy. It has been shown to inhibit the growth of the NSCLC cell line A549 as well as other cancers through inhibition of PI3k signaling.[130] It is also known to be an activator of peroxisome proliferator-activated receptor-γ (PPARγ) which can influence cellular metabolism and fatty acid oxidation [131, 132]. Finally, TS has also been used as a cardio protective agent to reduce the toxicity of anti-cancer therapies through an anti-inflammatory mechanism dependent on ANG II inhibition [133]. For these reasons, we hypothesize that a therapy combining AD with TS (AD+TS) will act synergistically to reduce both the viability and the CSC properties of NSCLC in the tumoroid model as well as in vivo models.

6.2 Results
IC₅₀ values were determined for AD in LLC1, A549 and H1299 cell lines cultured either as monolayer or tumoroids to ensure subsequent experiments were carried out using sub-lethal treatments. AD was found to be very toxic to these cells cultured as a monolayer with IC₅₀ values of 0.63nM (LLC1), 1.28nM (H1299), and 1.64nM (A549) (Figure 23A). Tumoroid culture was found to slightly raise the IC₅₀ values for all cell lines indicating that the tumoroid model better replicates the in vivo microenvironment where drug resistance is increased; with IC₅₀ values of 90nM (LLC1), 40nM (H1299), and 5nM (A549) (Figure 23B). TS alone was found to be non-toxic at the relevant therapeutic concentrations of 1-10µM (Figure 23C)
Figure 23. AD Reduces Viability in both Monolayer and Tumoroid Cultures of Lung Cancer Cell Lines

Dose response curves and IC50 values for mouse and human lung cancer cell lines cultured on monolayer (A) vs 3D scaffold (B). Cells were plated on scaffold in 96 well plates at a density of 5,000 cells per well. Serial dilutions of AD were added on day 4 and cell viability was assayed on day 6 by CellTiter-Glo. N=3. Cell viability was also assayed after 48hr treatment with varying concentrations of TS (C).
To test whether AD treatment reduces stem-ness in addition to reducing cell viability, a sphere formation assay was performed. AD was found to be able to reduce the sphere forming efficiency of isolated ALDH high cells from 4.8% in untreated cells down to 2.2% with only a 0.5nM AD treatment (Figure 24A). This indicates that AD is reducing the stemness characteristics of these cells. Because the CSC marker ALDH was found to be highly active in LLC1 cells cultured under low attachment conditions during the tumorsphere assay, we tested whether AD treatment would be able to reduce ALDH activity during tumorsphere growth. Indeed, treatment with as little as 3nM AD was able

Figure 24. Actinomycin D Reduces Sphere Formation Ability and ALDH Activity in LLC1 Tumoroids
A. Sphere formation efficiency of LLC1 sorted ALDH high cells with or without AD treatment (0.5nM) N=3*p≤0.05
B-C. ALDH activity of LLC1 cells cultured as spheres on low attachment plates with or without 3nM AD treatment (B) or as tumoroids on 3D scaffold after treatment with escalating doses of AD (C), as assayed by flow cytometry using ALDEFluor kit.
to reduce ALDH activity in tumorspheres from 64% to 13% (Figure 24B). LLC1 cells cultured as tumoroids on scaffold were also treated with AD in increasing concentrations (3-48nM), and a dose-dependent reduction in ALDH activity was observed (Figure 24C). Therefore, AD may be able to reduce the stem like properties of cancer cells as evidenced by its ability to lower ALDH activity and sphere formation.

![Figure 25](image)

**Figure 25. Actinomycin D Acts Synergistically with Telmisartan in Lung Cancer Tumoroids**

IC_{50} values were determined for cell lines cultured as tumoroids on scaffold LLC1(A) and, H460, H1299, A549 (B) treated with AD in the presence or absence of TS (10µM). Drugs were added on day 4 of culture and viability was assayed on day 6 using CellTiter Glo. Combination index was calculated using CompuSyn Software by comparing the effects of AD and TS alone vs in combination. IC50 and combination index values are provided in the associated table(C).

Additionally, TS was tested for its ability to synergize with AD to further reduce the viability of lung cancer cell lines treated with AD. LLC1 cultured on scaffold were treated with a combination of AD at varying concentrations plus 10µM TS. (Figure 25A) The addition of TS was found to reduce the IC50 of AD from 127nM to 52nM in a synergistic
fashion. (Calculated using CompuSyn software)(Figure 25A-C) This suggests that through the addition of non-toxic concentrations of TS a low dose of AD may be sufficiently effective. This effect was also seen in tumoroid cultures of human lung cancer cell lines (Figure 25B-C) indicating that the effects of AD+TS treatment are not cell line specific.

Next, an analysis was performed using Ingenuity Pathway Analysis (IPA) software aiming to predict mechanisms of interaction between AD and TS based on published findings present in the IPA database. (Figure 26) This analysis predicted 11 factors to directly interact with both drugs including caspase3, reactive oxygen species (ROS),

![Figure 26. Prediction of Mechanisms of Synergy Between AD and TS](image)

IPA was used to find molecules that are affected by both AD and TS. Direct interactions are highlighted in red, indirect interactions are shown in grey. CASP3, NOS2, and ROS were selected for further study.
and Nos2. We have already seen in chapter 4 how Nos2 is up-regulated in the tumoroid model so the fact that AD+TS are known to target it was encouraging.

The 3D tumoroid morphology previously described as a key feature of scaffold culture was assayed following drug treatment to determine if the treatments had any effect on the overall morphology of the cultures in addition to their ability to block the cells from acquiring stem-like properties. The combination treatment of AD plus TS was found to change the morphology of LLC1 cultured on scaffold. (Figure 27A) When cells were treated with TS or AD alone, they retained the same 3D tumoroid structure as untreated cells. However, when LLC1 were treated with AD+TS, the tumoroids were seen to break up, and more isolated single cells were visible. This change is indicative of the increased potency of the combination treatment since AD alone seems able to reduce stemness as assayed by ALDH activity; however, it is not able to break up the tumoroid structures at this low concentration without the addition of TS. Following observations of morphology, tumoroids from these treatment groups were assayed for ALDH activity. AD was found to greatly reduce ALDH activity which is consistent with previous experiments, however, AD+TS was able to reduce ALDH activity further, to 0% (Figure 27B). To investigate the mechanism of cell death occurring as a result of AD+TS treatment, a western immunoassay was performed to assay levels of PARP and caspase3, two proteins critical to promoting apoptosis. (Figure 27C) Following AD+TS treatment levels of both cleaved PARP and cleaved caspase3 were found to increase indicating that LLC1 tumoroids were undergoing apoptosis.
In addition to the ability of AD to reduce ALDH activity and sphere formation efficiency the effect of AD on several of the CSC related genes identified by the qPCR array were assayed. Scaffold cultured LLC treated with AD and AD+TS were found to have reduced expression of several stemness-related genes previously shown to have increased as a result of scaffold culture such as abcg2, dvl3, nos2, and plat. (Figure 28) The combination treatment reduced expression of all genes assayed with the exception of Oct4. TS alone was shown to reduce expression of CD44, Oct4, and Nanog revealing
that it may have broader anti-CSC effects than merely increasing the penetration of AD into tumors.

![Graph showing normalized fold change in CSC genes expression](image)

**Figure 28. Combination of Actinomycin D and Telmisartan Reduces Expression of CSC Genes in Tumoroids**

LLC1 cells were cultured as tumoroids and treated as in Figure 25. Cells were collected on day 6, RNA was isolated by affinity column method, and expression of CSC related genes were assayed by qPCR. Results were compared to LLC1 monolayer culture as control. N=3 *p≤0.05

To assay of the effectiveness of AD and AD+TS treatments in reducing tumor burden and CSC growth, these treatments were tested in multiple mouse models of lung cancer. Cells cultured in monolayer as well as CSC enriched scaffold cultures were tested to compare the effectiveness of these treatments on CSC specifically. One million LLC1 monolayer cells were injected subcutaneously into the flanks of C57BL/6 mice to initiate tumors. Starting on day 10-post injection, intratumoral treatments of AD (25µg/kg or 50µg/kg) were administered every 3 days for a total of 3 treatments. Tumor diameters were measured via caliper on days 11, 14, and 17. Only the 50µg/kg
treatment was found to significantly reduce tumor size, so this dose of AD was chosen for subsequent experiments. To determine the anti-tumor effect of TS this experiment was repeated with the addition of TS and AD+TS groups using only 50 µg/kg AD and 1 mg/kg TS. Both AD and the combination treatment reduced tumor size although the combination had a more significant effect while TS treatment did not reduce tumor size. (Figure 29A) We also tested the effectiveness of AD+TS treatment in a xenograft model using A549 subcutaneous flank tumors in Nod/SCID mice. In this model we also found that TS treatment has no significant effect on tumor growth, AD was able to slow tumor growth, but AD+TS resulted in the greatest reduction in tumor size. (Figure 29B)

In a second experiment, 100,000 3rd generation scaffold cultured LLC1 were injected subcutaneously into the flanks of C57BL/6 mice. These tumors were treated intratumorally with a vehicle, AD (50µg/kg), or AD plus TS (1mg/kg) every 3 days beginning on day 10. Using 3rd generation scaffold cells to initiate tumors provides a greater number of initial CSC meaning fewer cells are required for the tumor to grow compared to cells derived from monolayer culture. In these 3rd generation tumors, we observed a stronger effect of the AD+TS treatment as evidenced by the larger difference in size between AD+TS tumors and tumors in the other treatment groups. (Figure 29C).

To assess the stemness of the tumor cells following treatment, the tumors from the LLC1 scaffold group were collected; one tumor from each group was digested to form single cell suspension, and was assayed for ALDH activity by flow cytometry. The TS, AD, and combination tumors were all found to have reduced ALDH activity compared to
the control group. (Figure 29D) This indicates that AD and AD+TS treatments are effective at reducing CSC in addition to reducing overall tumor size and burden.

6.3 Discussion

Our results thus far have demonstrated a novel combination treatment (AD+TS) that is not only able to reduce the expression of stemness markers in lung cancer cells in vitro but also is able to reduce tumor growth in vivo. Using a novel 3D tumoroid model...
to enrich and maintain CSC in lung cancer cell lines has allowed us to study the gene expression changes that occur as CSC grow. We have thus identified the Wnt pathway as crucial for lung CSC development and re-confirmed the importance of known targets such as Sox2, ALDH, and Nos2. Using published literature to predict possible interactions between AD and TS we have identified and validated the apoptosis-inducing effects of AD+TS in lung cancer through a mechanism involving caspase-3 cleavage. Through this synergistic action, AD and TS are able to significantly reduce the viability of cancer cells both in monolayer and tumoroid culture while simultaneously reducing expression of CSC marker genes, ALDH activity, and interfering with the ability of cells to form 3D tumoroids. This synergistic action was validated in vivo where AD+TS was found to significantly reduce both tumor growth and stemness via reduction in ALDH activity.

While the mechanisms of AD’s anti-cancer activity are well known as stated in the introduction to this chapter, the use of TS in cancer therapy and specifically its combination warrant further study, some of which will be revealed in the following chapter. Because TS is a partial agonist of PPARγ, which causes metabolic changes within tumors to increase fatty acid oxidation possibly counteracting the Warburg effect it may play a secondary role disrupting the metabolism of CSC [131-133]. The use of metabolism to target CSC is a rapidly expanding area of research and TS’s role in this area warrants further investigation [59, 134, 135]. It has also been used to help reduce the toxicity of co-administered chemotherapeutics as its Ang-II inhibition has an anti-inflammatory effect that is cardio-protective indicating that the use of TS can have additional benefits outside the targeting of CSC [133].
CHAPTER 7

AD and TS Combination Treatment Reduces Stemness in Lung Cancer CSC through Modulation of the Wnt/β-catenin Signaling Pathway

7.1 Introduction

As described in chapter 4, the expansion of lung CSC observed in 3D tumoroid culture is associated with increased expression of several Wnt/β-catenin target genes (Sox2, ALDH, CXCR4, and Nos2). Therefore, in addition to the anti-ALDH and pro-apoptotic effects described in chapter 6 we hypothesized that combined treatment of AD and TS might be mediating its effects through alterations to the pro-CSC Wnt/β-catenin pathway.

Wnt/β-catenin is a core stemness pathway which along with Notch, sonic hedgehog, and Janus-activated kinase/signal transducer and activator of transcription (JAK-STAT), is known to regulate self-renewal and proliferation both in embryonic stem cells as well as CSC [24, 60, 136-138]. Its role in driving the growth and expansion of CSC has been extensively studied, and as mentioned in chapter 2, it is one of the pathways currently under investigation for novel inhibitors to block CSC expansion.

Wnt signaling involves a vast interconnected network of proteins that has classically been divided into canonical and non-canonical pathways. As research has progressed, increasing complexity has been revealed within the non-canonical pathways and they
can now be further broken down into three sub-categories; the planar cell polarity pathway, the Wnt/calcium pathway, and the non-canonical Wnt5/Fzd2 pathway [139]. (See Figure 30 for comparisons of canonical and non-canonical Wnt signaling pathways). Taken together these pathways are known to regulate expression of over 125 target genes [140, 141]. In general, Wnt signaling is initiated when one of the 19 known Wnt ligands binds to one of the ten isoforms of the frizzled receptor (FZD) in complex with the co-receptor LDL-related protein receptor 5/6 (LRP-5/6) on the cell surface [142]. This leads to the phosphorylation of LRP6 by GS3K/CK1γ and recruitment of disheveled (Dvl) which breaks up the β-catenin destruction complex.
(composed of GSK3β, APC, Axin, and Ck1α). This typically keeps cytoplasmic β-
catenin levels low by tagging it for proteasomal degradation. Therefore, when the
activity of the destruction complex is inhibited, β-catenin is allowed to accumulate in the
cytoplasm where it is stabilized and signaled to the nucleus by modifications such as
phosphorylation and acetylation. In the nucleus, β-catenin complexes with TCF/LEF
transcription factors to activate expression of their target genes.

Activation of non-canonical Wnt pathways can trigger distinct signaling events
depending on the specific Wnt ligand involved and the members of the receptor
complex that it binds to. For example, Wnt5 can bind to FZD in the absence of LRP5/6
resulting in activation of either PKC/NFAT (protein kinase C/ nuclear factor of activated
T-cell) or STAT3 target genes ultimately regulating cell adhesion and EMT.[142] Wnt5
can also bind to receptor tyrosine kinase-like orphan receptor 2 (ROR2) in the absence
of FZD resulting in the destruction of β-catenin and inhibition of canonical Wnt signaling
[143, 144].

We hypothesize that because the increase in stemness promoted by our tumoroid
model is associated with increased β-catenin activity, that the ability of AD+TS to block
stem-ness in this model may involve inhibition of the Wnt/β-catenin pathway. We further
hypothesize that because these drugs are not known to be direct inhibitors of any Wnt
signaling proteins, that their effects on the Wnt pathway may involve the production of
ROS and/or activation of PPARγ since these are known effects of the drugs.
7.2 Results

In order to get a better understanding of the broader effects of AD+TS treatment, we have performed RNA sequencing (RNA-Seq) on the drug-treated LLC tumor samples. Sequencing was performed, by our collaborators at the Beth Israel Deaconess Medical Center genomics core. 403 total genes were found to have either significantly increased or decreased expression in the AD+TS group compared to control. (Figure 31A) However, only 13 genes were found to be differentially expressed in all treatment groups exhibiting the same trend after AD, TS, and AD+TS treatment. (Figure 31B)

The two genes found to increase in expression were Dmd and Abcb1. While Dmd is the largest gene (containing 2.2Mb) indicating a possibility that its identification is an artifact of sequence alignment it could be involved in stem cell depletion as it promotes asymmetric division in muscle stem cells leading to differentiation [145]. Abcb1 encodes a bile salt transporter, which has also been known to transport drugs, so it may be upregulated as a survival response caused by drug treatment [146]. Among the decreased genes Lair1, Batf2, Gbp2, and Cxcl9 may have roles in stem-ness in this model. Lair1 is a cell signaling receptor typically found on hematopoietic cells however it is known to “exhaust tumor-initiating cells by apoptosis” in acute myeloid leukemia [147]. Batf2 promotes the formation of the β-catenin destruction complex resulting in GSK3β activation and β-catenin destruction [148]. Its expression may be decreased after treatment in an attempt to increase canonical Wnt signaling to support cell survival and proliferation. Gbp2 increases association between Dvl and GSK3β inhibiting GSK3β, therefore, a decrease in Gbp2 would reduce β-catenin activity by promoting its
phosphorylation by GSK3β [149]. This opposes the action of the Batf2 expression change indicating that the Wnt pathway may be dysregulated by our treatments. Cxcl9 is a chemokine that functions to attract T cells which is typically produced following interferon-gamma stimulation. It is also known to increase activation of β-catenin in B cell lymphoma [150].

![Diagram showing gene expression changes]

**Figure 31. RNA-Seq Reveals Gene Expression Changes that Result from AD and TS Combination Treatment In Vivo**

A. Gene Expression Changes in Treated LLC1 Tumors. RNA was isolated from drug treated LLC1 tumors (Figure 29C) and RNAseq was performed to identify differentially expressed genes within the 4 treatment groups. The number of genes identified as either increased or decreased in expression unique to each treatment or common to multiple treatments are shown.

B. Table depicting genes either increased or decreased in all (AD, TS, and AD+TS) treatment groups depicted in A.

Ingenuity Pathway Analysis (IPA) was performed using this data to identify downstream effects of the observed expression changes. We found the expression changes caused by AD+TS treatment to be consistent with decreased incidence of tumors, decreased migration of cells, and changes in glucose metabolism. (Figure 32A) This indicates that TS treatment may be affecting glucose metabolism in LLC tumors,
and is consistent with its well-known PPARγ agonism. IPA analysis was also used to predict which cell signaling networks are likely to be inhibited by AD+TS treatment using the RNA-Seq data. Figure 32B shows two of the top predictions based on differentially expressed genes in AD+TS tumors vs control. These networks predicted to be inhibited by AD+TS treatment rely on STAT1 and STAT3 signaling, a pathway known to interact cooperatively with Wnt/β-catenin [151-153].

![Figure 32. AD and TS Combination Treatment Reduces Tumor Growth, Migration, and STAT1/3 Signaling In Vivo](image)

IPA was used to predict the downstream effects of the genes found to be differentially expressed in the combination treatment vs control by RNA-Seq. **A.** depicts predicted downstream effects inhibiting tumor incidence, cell migration, and glucose metabolism. **B.** depicts predicted downregulation of networks in the STAT1/STAT3 pathway.
Since several differentially expressed genes identified by qPCR are involved in or targets of β-catenin signaling (Dvl2, Dvl3, STAT3, CD44, Oct4, Sox2) a TOPFLASH assay for activated β-catenin was performed to determine if β-catenin activity is being increased by scaffold culture and also if AD+ TS treatment is able to reduce β-catenin activity.

**Figure 33. β-catenin Activity is Increased in Tumoroid Cultures and Reduced by AD and TS Combination Treatment**

A. LLC1 cells were cultured as monolayer or on scaffold. On day 2 of culture cells were transfected with a β-catenin reporter plasmid and luminescence was assayed at the indicated timepoints (D3-7). LLC1 monolayer were also transfected with β-catenin reporter plasmid and treated with TS (1µM), AD(2.5nM) or combination after 24hr. Luminescence was assayed at 72hr. N=3 *p≤0.05. B. Relative β-catenin abundance and phosphorylation in drug treated LLC1 tumors was assayed using a Phospho antibody array (Full Moon Biosystems). Relative normalized fold change was calculated using Graph Pad Prism software. One way ANOVA was used to determine significance. N=6 *p≤0.05
activation. LLC1 cultured on scaffold were shown to have higher levels of active β-catenin than LLC1 monolayer cultures (Figure 33A). Also, monolayer LLC1 cultures treated with AD + TS had significantly reduced levels of active β-catenin (Figure 33A-B). This finding is significant because β-catenin activity is known to drive both metastasis and the CSC phenotype in breast cancer, colon cancer, and hepatocellular carcinoma and because there are currently no drugs approved to target the Wnt/β-catenin pathway.(25-29) In addition, AD+TS treated tumors were found to express lower levels of both total and active phospho-β-catenin (Figure 33B).

To further investigate the effects of the combination treatment on the Wnt/β-catenin pathway we performed an analysis of protein phosphorylation on drug-treated LLC1 tumor samples using an antibody array chip (Full Moon Biosystems). Overall activation of the Wnt pathway was reduced by the combination treatment and several key changes to the pathway were observed including decreased production of Wnt 5a and decreased activation of CaMk2 and CKI gamma indicating that the treatment may be affecting the Wnt/calcium pathway in addition to the canonical Wnt/β catenin pathway (Figure 34). Downstream effectors of the Wnt pathway were also found to be reduced following AD+TS treatment including phospho-MAPK, phospho-Src, and phospho-AKT indicating that the processes of cell growth and proliferation controlled by these pathways may be reduced by AD+TS treatment. This finding corroborates the RNA-Seq results shown in figure 32, which indicated decreased cell proliferation in LLC1 tumors following AD+TS treatment.

To confirm that the canonical Wnt pathway is inhibited by AD+TS treatment we performed western immunoassays for the Wnt ligand binding co-receptor LRP6.
Tumoroid culture was found to increase both the expression and phosphorylation of LRP6 compared to monolayer culture in LLC1, and AD+TS treatment was found to inhibit LRP6 expression. (Figure 35A-B). This was repeated in protein isolated from drug-treated LLC1 tumors to confirm the phenomenon in vivo, and indeed total LRP6 was found to be lower in the AD+TS group (Figure 35C). Phospho-LRP6 was not observed in tumor samples possibly due to a limitation of the antibody or lower abundance of the phosphor-protein in vivo.
**Figure 35. Expression and Activation of the Wnt Co-Receptor LRP6 is Reduced by AD and TS Combination Treatment**

Expression of the frizzled co-receptor protein LRP6 was determined by western immunoassay in LLC cell line (A) and tumor (C) samples. In the LLC cell line levels of active phospo-LRP6 was also determined (B) and tumoroid groups were compared to monolayer as a control. Cells were treated with 10µM TS, 48nM AD or the combination for 48hr. Assays were performed using the Wes system (Protein Simple).
The IPA analysis also predicted AD and TS to interact via generation of reactive oxygen species. (ROS) Therefore we used a ROS indicator dye (CM-H2DCFDA) to determine whether ROS production is increased in LLC1 following treatment. Figure 36 shows that TS is a potent activator of ROS resulting in a 19% increase after 1µM treatment. When combined with a low dose of AD (0.4nM), which is only able to generate a 5% increase on its own, the AD+TS combination worked synergistically to cause an almost 30% increase in ROS.

Figure 36. AD and TS Combination Synergistically Increases Production of ROS

Production of ROS was assayed in LLC1 monolayer cultures treated with 0.4nM AD, 1µM TS, or the combination for 48hr. The dye CM-H2DCFDA was used as an indicator of general oxidative stress. Staining was assessed using fluorescent microscopy (B) while brightfield images were also collected for each field as a reference (A). Staining was also assayed by flow cytometry using the FITC channel (C).
7.3 Discussion

A significant *in vivo* mechanistic finding of our studies is that the efficacy of the novel AD+TS combination treatment approach involves anti-CSC effects, which are at least partially mediated by alterations in the Wnt/β-catenin pathway, leading to decreased activity of key cell proliferation signals and ultimately apoptosis through a mechanism involving increased ROS production and caspase-3 cleavage. This finding is significant in part because as discussed in chapter 4, expansion of CSC in the lung tumoroid model coincided with increased β-catenin transcriptional activity and expression of Wnt/β-catenin target genes highlighting the importance of this pathway driving the acquisition of the CSC phenotype. Indeed the enhanced cell-cell and cell-scaffold interactions promoted by tumoroid growth may be crucial to initiating this β-catenin activation either by signaling through the cell adhesion molecules E-cadherin or N-cadherin or through the rearrangement of adherens junctions, but more work is needed to determine the validity of this hypothesis.

By combining the techniques of RNA-Seq and phosphor-protein analysis, we have profiled the effects of AD+TS treatment on the Wnt/β-catenin pathway as well as its downstream effects on cellular properties such as migration, proliferation, and tumor growth. Wnt5a, which we have found to be elevated in LLC1 tumors and which was reduced by our combination treatment, has been reported to act as either a tumor promoter or tumor suppressor depending on the cancer type [154]. In NSCLC it has previously been associated with tumor cell proliferation and expansion of CSC [155,
This diverse activity may be attributed to the fact that Wnt5a can either activate or inhibit β-catenin phosphorylation through the canonical Wnt pathway depending on the cellular context. Its function depends on its binding to ROR1/2 (receptor tyrosine kinase-like orphan receptor) in the presence or absence of frizzled [157]. When Wnt5a activates β-catenin after binding to the FZD-4/5 LRP5/6 complex, downstream PPARγ activation is suppressed, and β-catenin/TCF target genes are transcribed [155, 158]. Wnt5a can also suppress PPARγ activation through the alternative Wnt/calcium pathway through a calmodulin-dependent mechanism involving NLK [158]. In this way, activation of canonical Wnt or Wnt/calcium signaling which lead to cell proliferation and acquisition of CSC traits can be linked to a decrease in PPARγ activity [159].

Alternatively, when Wnt5a binds to ROR2 in the absence of FZD, β-catenin activation is inhibited. TS is known to activate PPARγ as a partial agonist, which can, in turn, inhibit activation of β-catenin either by stabilizing the APC/GS3kβ destruction complex or by inhibiting the translocation of β-catenin to the nucleus [160-163]. This mechanism has been shown to be involved in the differentiation of MSCs into osteoblasts [158]. The inhibition of β-catenin activation in this manner not only reduces its pro-proliferation signal but also reduces the cell’s ability to repair the DNA damage caused by AD treatment [164]. It is known that inhibition of the Wnt/β-catenin pathway can promote DNA damage in the presence of the genotoxic chemical benzo[a]pyrene, so it is likely to also promote DNA damage in the presence of genotoxic concentrations of AD [165]. In addition to inhibiting transcription, AD can also cause double-stranded breaks in DNA leading to increased levels of ROS, which in turn can enhance apoptosis [166-168]. This may further contribute to the apoptosis-inducing synergistic action of TS.
in the combination treatment since other PPARγ agonists have been demonstrated to increase ROS levels in lung cancer cells by altering glucose metabolism to reduce glutathione levels ultimately resulting in cell cycle arrest [169]. This effect appears to be dependent on TS’s activation of PPARγ and therefore distinct from its angiotensin inhibition dependent anti-inflammatory, cyto-protective role.

The dual stimulation of ROS production that occurs as a result of the combination treatment may play a key role in the treatment’s ability to precisely target CSC through the Wnt pathway, since ROS themselves can regulate the transcriptional activity of β-catenin [170]. Our data show that the combination of genotoxic AD with TS’s multiple effects regulating metabolism and inflammation is able to reduce tumor burden and simultaneously reduce expression of CSC markers in both in vitro and in vivo models of lung cancer. TS is known to induce ROS production via activation of PPAR gamma, and TS is also known to inhibit JAK/STAT signaling[171]. TS alone has been reported to induce apoptosis in some cancer cell lines including prostate and colon, but conflicting reports suggest that this effect is not universal to all cancers. [172, 173]. We have implicated Wnt signaling as a target of this treatment and reported the treatment’s effects on both the canonical and Wnt/calcium pathways. Future directions will include further investigation into the regulatory mechanism by which changes in ROS and PPARγ activity induced by these drugs are able to alter the transcription and function of Wnt target genes to reduce the stemness of CSC.

In sum, our results thus far have demonstrated that CSCs expand in lung cancer tumoroids and tumors wherein the Wnt signaling pathway plays a crucial role in lung CSC development, and this signaling involves several important known targets such as
Sox2, ALDH, and Nos2. Further, novel combination treatment was designed using two known FDA-approved drugs, AD and TS, which not only reduced the expression of stemness markers in lung cancer tumoroids *in vitro* but also was able to reduce tumor growth *in vivo*. Moreover, the pivotal role of the Wnt signaling pathway was demonstrated in the synergism underlying the combination therapy.
CHAPTER 8

Conclusion

In conclusion, we have demonstrated and validated a novel in vitro model for lung CSC culture and successfully used this model to identify both a novel pathway regulating CSC growth (NPRA) and a novel treatment targeting CSC (AD+TS). To our knowledge, our lab is the first to demonstrate the importance of NPRA signaling in CSC development linking it with increased self-renewal and tumor initiation. We are also the first to demonstrate the effectiveness of the AD+TS treatment both in vitro and in vivo targeting CSC and reducing tumor burden through a Wnt/β-catenin dependent mechanism.

In chapter 4 we provided evidence that our 3D tumoroid model truly enhances the growth of CSC in lung cancer cell lines and biopsies finding a positive correlation between tumoroid growth and expression of the CSC markers including ALDH, CD44+/24-, and the transcription factors Sox2, Oct4, and Nanog. Also, we established that CSC within tumoroids identified by these markers were able to initiate tumors in vivo at low cell concentrations validating their status as true CSC. Through examination of the gene expression changes that occur in the tumoroid model, we identified Wnt/β-catenin signaling as a crucial pathway promoting lung CSC growth. This pathway has been previously described as a promotor of CSC in multiple cancers including lung, colon, and hepatocellular carcinoma lending support to the validity of the tumoroid model.
In chapter 5 we have shown how the tumoroid model can be used to enrich CSC in culture in an effort to discover novel pathways regulating their growth. We have identified that the expression of NPRA is correlated with CSC marker expression and that inhibition of NPRA signaling impairs the ability of lung CSC to self-renew and to initiate tumors. To our knowledge, this represents the first implication of the NPRA pathway as a driver of stem-ness in cancer revealing NPRA as a novel target for future studies aiming to develop treatments targeting CSC.

Figure 37. Summary of changes to the Wnt pathway as a result of AD+TS Treatment
In chapter 6 we demonstrate the effectiveness of the tumoroid model though validating by screening for CSC targeted drugs. We show that cells grown as tumoroids are more resistant to drug treatments and thus are a better replication of *in vivo* tumors. We have characterized the effects of our novel combination treatment (AD+TS) and its ability to decrease lung cancer tumoroid viability, ALDH activity, self-renewal through sphere formation, and even formation of the tumoroids themselves resulting in the induction of apoptosis through cleavage of caspase-3. The anti-CSC action of the AD+TS combination treatment was further validated using both an in vivo syngenic model and an in vivo xenograft model where it decreased tumor size and growth rate in addition to ALDH activity.

Finally, in chapter 7 we investigated the molecular mechanisms responsible for AD+TS’s ability to target CSC and the involvement of Wnt/β-catenin signaling. We began by surveying the gene expression changes caused by AD+TS treatment in vivo using RNASeq which confirmed our in vitro results demonstrating that AD+TS treatment reduces tumor incidence. We observed a decrease in active β-catenin following AD+TS treatment in monolayer, tumoroid, and *in vivo* tumors as well as signaling intermediaries downstream of β-catenin including phospho-AKT, and phospho-MAPK. We also identified decreased activation of an upstream activator of β-catenin, LRP6, further supporting the downregulation of canonical Wnt/β-catenin signaling. A summary of these effects can be seen in figure 37. Additionally, ROS generation was increased by AD+TS treatment, which offers a possible mechanism for the observed induction of apoptosis and changes in Wnt signaling, but further investigation is needed in this area.
Taken together, the results presented in these studies have highlighted the importance of NPRA and Wnt/β-catenin signaling in the acquisition of CSC traits in lung cancer. The knowledge acquired from this study could be useful in the future to develop cancer therapies targeting CSC, and ultimately improving patient outcomes.
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Appendices
Appendix A: IACUC Approvals

MEMORANDUM
TO: Subhra Mohapatra,

FROM: Farah Mouvi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 3/11/2017
PROJECT TITLE: Xenograft mouse models of cancer
FUNDING SOURCE: USF department, institute, center, etc.
IACUC PROTOCOL #: F150003403
PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 3/11/2017:

Mouse: C57BL/6j (6-8 wks/male-female) 300
Mouse: BALB/c (6-8 wks/female) 90
Mouse: NOD.CB17-Prkdcscid/J (6-8 wks/male-femail) 882
Mouse: 021837/NOD.Cg-Pkd1d1I2rg Tg (CAG GFP) 102/24 (5-6, male-female) 324

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• All costs invoiced to a grant account must be allocable to the purpose of the grant. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other
MEMORANDUM

TO:                  Subhra Mohapatra,

FROM:               Farah Moulvi, MSPH, IACUC Coordinator
                    Institutional Animal Care & Use Committee
                    Research Integrity & Compliance

DATE:               3/17/2014

PROJECT TITLE:      Xenograft mouse models of cancer

FUNDING SOURCE:      USF department, institute, center, etc.

IACUC PROTOCOL #:    R IS00000506
PROTOCOL STATUS:     APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 3/17/2014:

Mouse: C57BL/6 (6-8 weeks) 349

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol. Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.

• All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.