Tumor Induced Cardiovascular Dysfunction

DISSERTATION

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By
Raymond David Devine
Graduate Program in Molecular, Cellular and Developmental Biology

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Dissertation Committee:
Loren E. Wold, Advisor
Peter J. Reiser
Denis Guttridge
Martha Belury
Abstract

Cancer-induced cachexia is a syndrome of marked adipose and skeletal muscle wasting that is a complication for approximately half of cancer patients. The wasting effects of cachexia on these tissues have been well recognized; however, the impact of cachexia on cardiac muscle has only recently been investigated. An important component of cardiac integrity is the extracellular matrix (ECM), which provides physical support for the heart. Disruption to the ECM is a shared feature in several cardiac diseases. Changes to the ECM are caused by matrix metalloproteinases (MMPs). MMPs are enzymes that degrade the ECM, altering the structure of the heart. MMPs have been implicated in different cardiac diseases; however, their presence during cancer-cachexia had not been investigated. We utilized the c26 model, a colon carcinoma cell line, subcutaneously implanted in CD2F1 mice to induce cachexia. Mice were euthanized twenty-one days after injection, losing approximately 10-15% of their lean body mass. The mRNA and protein results in the heart showed significantly increased amounts of MMPs. MMP activity is associated with fibrosis, which corroborated to significantly increased collagen deposition in the hearts of tumor-bearing mice compared to controls as visualized by Picrosirius-Red staining. In order to examine MMP contribution, we utilized a pharmacological MMP inhibitor, minocycline.
Minocycline inhibits a broad spectrum of MMPs. When compared to untreated tumor-bearing mice, minocycline treated mice had significantly improved cardiac function. Minocycline treatment decreased only the mRNA expression of MMP-9 in the panel of MMPs. The mRNA expressions of collagen I and III were also significantly decreased with treatment. Minocycline appears to correct cardiac function through the inhibition of MMPs without decreasing expression, representing a possible therapeutic.

A proteomics study of hearts from c26 tumor-bearing mice showed metabolic differences implicating Hypoxia-Inducible Factor-1α (HIF-1α). HIF-1α is a transcription factor regulating over a hundred genes in response to hypoxic conditions. We observed an approximate two-fold increase in the hearts of tumor-bearing mice. HIF-1α is increased in hypoxic conditions, and examining the arterial blood of tumor-bearing mice found significantly decreased oxygen pressure and concentration when compared to control mice. Increases in HIF-1α are associated with lipid accumulation, we used Oil Red O staining to look at lipid deposition of tumor-bearing mice. We found significantly increased lipids in the hearts of tumor-bearing mice, indicating HIF-1α was causing metabolic changes in the heart. The role of HIF-1α requires extensive investigation to determine its role in cancer cachexia.

Lastly, we examined the effect of calcium handling and sarcomeric proteins on contractile kinetics in the hearts of tumor-bearing mice. We found significantly increased phosphorylated Ryanodine receptor (RyR) with significantly decreased amounts of FKB12, a protein that stabilizes RyR calcium pores. We also found significantly increased L-type Calcium Channel. Unlike other studies we did not find a decrease in
troponin I or a MHC isoform shift in the heart of tumor-bearing mice. This would indicate a leaky calcium channel but further work is necessary to determine how these may be contributing to cardiac dysfunction in cancer cachexia.

Our findings demonstrate new pathways contributing to cardiac dysfunction in addition to the ubiquitin proteasome pathway, which is regarded as the cause of most wasting effects during cachexia. Overall our results are preliminary and new not only in cancer cachexia, but cancer as well. MMPs and HIF-1α have not been shown to be increased in any tissue aside from the tumor in cancer studies. Further research is required to determine how our findings impact cardiac function and metabolism during the course of cancer cachexia.
Dedication

This document is dedicated to my friends and family.
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Vita

January 6, 1986……………………………..Born, Columbus, Ohio

2004……………………………………………..St. Charles Prepatory

2008……………………………………………..B.S. Biology, Ohio Dominican University

2008……………………………………………..B.S. Chemistry, Ohio Dominican University

2008-2010 .................................................Scientific Information Analyst, Chemical

Abstract Services

2010-Present………………………………..Molecular, Cellular, and Developmental

Biology degree program, The Ohio State

University

Publications

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Chapter 1: Introduction to Cancer Cachexia, Heart Failure, and Tumor Induced Cardiovascular Dysfunction

1.1 General Introduction

Cancer is the uncontrolled cellular division and differentiation that results often in a solid neoplasm, referred to as a tumor. While the effects of cancer can be detrimental on their own, cancer patients are at risk of developing cancer-induced cachexia. Cachexia is broadly characterized as the marked loss of body mass through the depletion of skeletal muscle and adipose tissue. This loss of tissue is thought to be the result of increased protein breakdown and decreased protein synthesis, altering the carefully maintained metabolic state (1, 10, 135). Preliminary results from human and experimental models of cancer cachexia have revealed cardiac complications as a result of tumor burden (127). While there have been no cardiac function experiments performed in humans, histological evidence from patients who died as a result of cachexia showed significant fibrotic remodeling in the left ventricle. The role of cancer as well as cancer-induced cachexia will be explained in further detail, as well as the current mechanisms of cancer cachexia. While the mechanisms of cardiac dysfunction in humans and experimental
models are currently, unknown the mechanisms of cancer cachexia can be inferred to possibly explain cardiac dysfunction which will be detailed in this work.

1.2 Cancer

1.2.1 History of Cancer

Cancer has been a recognized disease since antiquity, with descriptions of it being found in papyrus medical texts as early as 1600 BC. The word cancer is derived from the Greek καρκίνος; meaning crab which is how the solid tumor appeared to Hippocrates when he described it. Tumors were originally thought to be the result of an imbalance of humors in the body and were primarily treated only surgically. During the 16th and 17th centuries, when it became acceptable for doctors to dissect corpses to determine a cause of death, it was discovered that tumors could be present inside the body. It was originally believed that clogged milk ducts are what led to the development of breast cancer and were still thought to be a result of humoral imbalances. At the start of the 20th century, the cell theory had become more recognized and it was discovered that cancer was the result of uncontrolled cell growth. While the causes were still unclear, it was actually a British surgeon, Percivall Pott, who described, in the 18th century that occupation may play a role in tumor development, as chimney sweeps at the time were diagnosed with a high incidence of cancer. In modern times, cancer has been recognized as a combination
of environmental as well as genetic factors and the treatment methods have improved (35, 153).

### 1.2.2 Treatment of Cancer

Cancer treatment currently has three main components; surgical, chemotherapeutics, and radiation. At the onset of cancer, typically biopsies will be taken of the solid mass, or the tumor, in order to determine what type of cancer it is and the best treatment method. Surgery is the most common method of dealing with solid tumors that have not metastized, or spread to different locations, and is one of the most tolerated (145). The procedure involves cutting open the patient in an approximate location to the tumor and carefully removing the solid mass tumor, and complications can arise if the entire tumor is not carefully removed as the cells will regrow to form another solid mass. Depending on the size of the tumor there may be significant numbers of blood vessels leading directly into the tumor complicating the surgery due the possibility of hemorrhagic shock. For this reason, surgery is often combined with chemotherapy in an effort to reduce the tumor size to an operable level. Chemotherapy involves the administration of chemicals directly to the patients’ bloodstream. While there are several groups of chemotherapeutics available, they all share the end goal of killing rapidly dividing tumor cells (17). The limitation to chemotherapeutics is their cytotoxicity, which while beneficial for removal of cancer cells, is also detrimental to the healthy cells of the body. Highly replicating tissue, such as the intestine, are especially at risk during
chemotherapy. This is often why cancer patients commonly experience lack of appetite, malnourishment and may contribute to the anorexia/cachexia development. The effects of chemotherapeutics are not limited to rapidly dividing cell populations. A common chemotherapeutic, Doxorubicin, has been shown to have potent cardiotoxic, effects correlating with dose, and can lead to heart failure (126, 148). In order to bypass these systemic effects, radiation is used where possible which depends on the location and size of the tumor. Radiation therapy works by damaging the DNA directly through the action of ionizing radiation which breaks apart the base bonds and disrupting the helical structure of DNA. In early therapies, ionizing radiation was administered in specific areas due to the lack of the ability to focus the radiation. Naturally, as the ionizing radiation passed through the skin and other tissues, it would scatter, leading to indirect radiation damage of the surrounding tissue. Currently, through the use of parabolic devices, the administration of radiation has become more specific and well tolerated. There are new methods being developed constantly in the hopes of limiting the damaging effect of chemotherapeutics by introducing tumor-targeted molecules or physical attractors such as nanowires that will attract specially encapsulated chemotherapeutics. The development of better cancer treatments will hopefully lead to the decrease of cancer mortality worldwide.
1.2.3 Epidemiology of Cancer

The tracking and statistical analysis of cancer is a relatively recent occurrence in the history of cancer. Despite being known since 1600 BC cancer has not been systematically tracked until recently. Even with the recognition of cancer as a treatable disease with the humoral theory most treatments were conducted at home, not under hospital supervision. After the development of the cell theory, there was a push to standardize cancer treatment and have treatment occur in the hospital. With treatment occurring at the hospital records were finally available for widespread use and the epidemiology of cancer was able to be determined. Currently, cancer is the second leading cause of mortality in the United States behind cardiac disease; in the United Kingdom, it has risen to the leading cause of mortality, with cardiovascular disease being second (121, 137). Monitoring in developing countries is still highly variable due to the lack of systemic medical infrastructure. Cancer is predicted to rapidly increase in third world and developing countries as the management of infectious diseases is increased. In the United States approximately 1 in 3 people will be diagnosed with cancer at some point in their lifetime. It has become evident that, as age increases, the chances of cancer rises with over 78% of cancer diagnoses occurring in those 55 years and older (121). In the United States cancer accounts for approximately 25% of deaths, with approximately a third being from lung disease. Despite cancer treatments the number of deaths has remained largely steady. This is primarily due to increased life expectancy, which contributes to more cancer cases causing the percentage of cancer deaths to remain
relatively stable. With worldwide levels of human health indices increasing, cancer rates and mortality are expected to increase sharply, driving the need for more comprehensive monitoring and treatment.

1.3 Cachexia

1.3.1 Wasting condition

Cachexia is a term used to describe the marked wasting of skeletal muscle and adipose tissue during a disease state. Cachexia can be brought about by progressive diseases, such as chronic obstructive pulmonary disorder (COPD), and heart failure but also by more acute diseases such as cancer and burns. For the purpose of this writing, only cancer induced cachexia will be considered and its mechanisms in relation to cardiac dysfunction. Cancer induced cachexia occurs in approximately half of cancer patients and will be the primary cause of death for a quarter of those affected by it. The cause of death due to cancer cachexia is primarily respiratory failure, where the oxygen levels can no longer be maintained (110). Cancer cachexia, as mentioned previously, is defined by a marked reduction in the adipose tissue and skeletal muscle. There are no accepted clinical guidelines to determine cachexia but the common way to determine if a patient is experiencing cachexia is if they lose 5% of their premorbid body mass over the course of six months (31). Cachexia is difficult to establish guidelines for because during the course of common cancer treatment, chemotherapy, radiation, and surgery, generally
cause weight loss or contribute to decreased food intake on their own. Commonly, the weight loss attributed to cancer treatments is anorexia, which differs significantly from cachexia. Anorexia differs from cachexia in its mechanisms, treatment, and causes. Anorexia experienced by cancer patients is often the result of appetite suppression due to chemotherapeutics as well as reduced gut motility (21). The wasting effect of anorexia differs from cachexia as well; in anorexic patients, adipose tissue is often depleted first, followed by skeletal muscle, while in cachexia, they are simultaneously broken down. A marked difference between anorexia and cachexia, however, is that anorexia can be nutritionally reversed while cachexia cannot (44). Cachexia, in broad terms, is an imbalance between the synthesis and degradation of proteins. In conditions such as anorexia, or starvation this imbalance is due to protein breakdown to supply energy to the body in order to sustain life function. This imbalance can be restored by the administration of caloric dense food, sparing protein breakdown in the body. In cachexia, however, the administration of caloric dense food has been shown at the minimum to maintain body mass and prevent further weight loss but does not improve body mass. The mechanisms behind the shift in protein degradation will be discussed further in the mechanism section.
1.3.2. Depressive Condition

While the wasting condition is the most evident and concerning of cancer cachexia, depression has become a recognized effect of cachexia and contributes to patient morbidity. Depression, as a psychiatric symptom, is characterized by increase anxiety, loss of appetite, and fatigue among other symptoms (21, 56, 87). In the setting of cancer, there are innate and extrinsic complications contributing to depression. Part of the problem with diagnosing depression in cancer patients is that some of the symptoms, such as tiredness and anxiety, are considered common complications of the disease itself. Previous studies have been criticized for using terminally ill and bed-ridden cancer patients, which have markedly increased percentage of depression compared to the normal population. More recent studies looking at cancer patients receiving treatment but not bed-ridden found double the percentage of patients experiencing symptoms compared to the general population (88, 90). Depression was found to be more common in cancer patients who experienced pain due to their condition over those who did not. The depression associated with pain was found to reinforce pain, which then further reinforced the depressive phenotype, creating a vicious cycle (34, 39, 67). Depression is becoming a more recognized complication of not only cancer but cancer cachexia, as well. Studies have shown that there are increased amounts of inflammatory cytokines in the brain contributing to this depression (20). In addition to depression, inflammatory cytokines can also cause decreased appetite, contributing to the cachectic phenotype. Cachectic patients often experience frustration at the involuntary weight loss that’s
coupled with their lack of appetite for food. This can cause a cycle worsening the cachectic patients mood and outlook, with frustration directed at themselves and at others. While currently little is known about the exact mechanisms of depressive mood, it needs to be explored so that effective therapies can be developed to treat the depression associated with this syndrome.

1.3.3. Treatment of Cancer Cachexia

Treatment of cancer cachexia, in large part, is focused on the prevention of cachexia through treatment of the initial insult. For instance, in cancer cases there is treatment with chemotherapy, radiation, and surgical methods to remove or eliminate the cancer before it can trigger cachexia. These methods, however, do not necessarily prevent cachexia in all cases and can sometimes be contributing factors. Once cachexia sets in, the patient becomes refractory to conventional cancer treatments, becoming less responsive as well as tolerating chemotherapy poorly. Due to the multimodal nature of cachexia, with several signaling pathways being activated, there is currently no standard care when cachexia occurs. When cachexia was first being described as a symptom, it was thought that increased nutritional support would reverse or slow cachexia. This was found to be inadequate to prevent weight loss in cachectic patients (4). Even further advancements in nutritional support, with the product Jevity containing 1.5 calories/ml, has been shown to only stabilize current weight loss but not restore previous weight loss.
While there have been advancements in understanding the mechanisms of cancer cachexia and close collaboration between basic and clinical sciences, the effective treatments for cancer cachexia are still in preliminary stages. Despite strong experimental evidence, some treatments have produced disappointing results. Bortezomib is a proteasome inhibitor and was one of the first to be tested in human clinical trials. While it has been shown to be effective in the treatment of multiple myeloma, possibly as a result of the inhibition of breakdown of pro-apoptic factors into active apoptic factors, was shown to be ineffective in a trial with pancreatic patients (59). Cannabinoids, marijuana derivatives, while being effective at increasing the appetite and caloric intake of AIDS patients, have been shown to be less effective when administered to cancer patients (99). Not all treatments have proven to be ineffective in clinical trials, however. Cyclooxygenase-2 (COX-2) inhibitors have been shown to reduce experimental models of cachexia, possibly by suppressing systemic inflammation. In a trial with head and neck or gastrointestinal cancer, the COX-2 inhibitor, celecoxib, was shown to improve weight and body mass index when compared to placebo (82, 83). Ghrelin and ghrelin mimetics have also shown promise in early clinical trials. Ghrelin is a naturally occurring peptide that increases appetite and decreases sympathetic nervous activity. Ghrelin administration, however, has been shown to be ineffective when administered in single doses intravenously. When given over the course of several weeks consistently, it has been shown to improve weight and body mass index, indicating it is a possible therapeutic and seems to be well tolerated over long periods of time (36, 37). As more mechanisms of cachexia are uncovered, they will need to be investigated in a clinical
setting since, despite strong experimental evidence, not all drugs are effective in patient treatments.

### 1.3.4 Epidemiology of Cancer Cachexia

Cancer cachexia is difficult to study because until recently there were no standard measures of clinical cachexia. With increased attention and monitoring of cachexia, there are now measurements of cachexia incidents. Patients who develop cancer have approximately a 50% chance to develop cachexia overall. Depending on the location of the cancer, there are disproportionate amounts of cachexia. Lung, gastrointestinal, and pancreatic cancers have high occurrences of cachexia (134, 135). These particular cancers may have increased incidents of cachexia due to their location in relation to important anatomical structures. Lung cancer patients often have difficulty with oxygen transfer and this may contribute to the increased systemic inflammatory condition that contributes to cachexia. The gastrointestinal and pancreatic cancers may involve the blockage of the intestines preventing adequate nutrient absorption contributing to the cachectic state. Patients who develop cachexia are at risk of increased mortality and further complications due to the weight loss. Cachexia will be the primary cause of cancer deaths in 25% of patients who develop cachexia. The primary cause of death as the result of cachexia is from respiratory failure.
1.4 Heart Function

Before the impact of cancer on cardiovascular function can be discussed, cardiac function in general must be discussed. The separation and distribution of deoxygenated and oxygenated blood is accomplished through a chamber system. The heart has four chambers in total, two atria, and two ventricles. The right atria and ventricle are responsible for handling deoxygenated blood coming through the superior and inferior vena cava. This deoxygenated blood is shunted to the pulmonary artery to be reoxygenated by the lungs. This now oxygenated blood returns through the pulmonary vein through the left atrium. From the left atrium the blood passes through the mitral valve into the left ventricle where it is expelled through the aortic valve into the aorta. Through this pumping action oxygenated blood is distributed to the rest of the body through the arteries. When this pumping action is diminished or disrupted it becomes known as heart failure. Heart failure can arise through multiple etiologies but the end result is the same, oxygenated blood can no longer be adequately distributed throughout the body. The loss of function is usually due to compromise of the left ventricle, which expels oxygenated blood, and warrants a special discussion of the contractile mechanism.
1.4.1 Contraction, Relaxation; Cross Bridge Cycling

In order to provide oxygenated blood, the left ventricle contracts forcing quick emptying of the chamber, pushing blood through the aorta to the rest of the body. This contraction is accomplished by shortening of the contractile apparatus. A single unit of the contractile apparatus is referred to as the sarcomere, and represents the functional unit of contraction. A sarcomere consists of a thin filament and a thick filament contained between two Z-lines, the distance of which is 2.2 um in humans. The thin filament consists of actin which is covered by tropomyosin, which is further bound by Tropnin (Tn) complexes. The thick filament consists of the ATP-dependent protein myosin, which is the motor protein for contraction. The Tn-tropomyosin complex is necessary for proper contraction and relaxation. With the Tn-tropomyosin complex myosin would bind with high affinity to actin. The Tn complex consists of three proteins: TnI, TnC, and TnT. These proteins bind to tropomyosin allowing it to cover myosin sites on actin. As calcium is released (see 1.5.2 below for more information), it binds to TnC which causes a conformation change in the Tn complex, shifting tropomyosin away from the myosin binding sites. The myosin head hydrolyzes ATP, resulting in an ADP+Pi (inorganic phosphate) complex allowing the myosin head to bind to actin. After binding to actin the release of ADP+Pi from the myosin head initiates the power stroke. This causes the actin filament to slide forward, bringing the Z-lines closer together, resulting in contraction. In the heart, this contraction is referred to as systole, and the inverse event is diastole.
In diastole, the myosin head must be released from actin in order to achieve relaxation. ATP binds to the myosin head, moving the myosin head back into the ‘cocked’ position and releasing the actin-myosin complex. Calcium is also brought back into the sarcoplasmic reticulum. Without calcium binding to the Tn complex any longer it reverses the conformational change bringing the Tn-tropomyosin complex back over the myosin binding sites on actin. With the myosin binding sites blocked, the actin filaments return to their resting position and the Z-lines are pushed back out to their original state. In the heart, this relaxation allows the ventricles to begin filling with blood in preparation for the next contraction.

1.4.2 Excitation Contraction Coupling

Calcium is a crucial ion for contraction. The heart has developed mechanisms to deal with calcium handling that differ slightly from those in skeletal muscle. In cardiac muscle, the L-type calcium channels are not physically coupled to the Ryanodine Receptor (RYR). Instead the heart uses a mechanism, referred to as calcium induced calcium release. An action potential generated by the sinoatrial node causes calcium to enter through the L-type calcium channels. Calcium then interacts with the RYR rapidly releasing calcium from the sarcoplasmic reticulum. Once calcium is released from TnC, it must be quickly removed from the intracellular space back into the sarcoplasmic reticulum. Returning calcium into the SR is achieved through specialized proteins, Sarco/Endoplasmic Reticulum ATPase (SERCA) and phospholamban (PLN). SERCA
brings calcium back into the SR through an ATP-dependent mechanism since ATP hydrolysis is required to move calcium against the gradient. SERCA is modulated by the regulatory protein, PLN. PLN is normally inhibitory, preventing calcium movement through SERCA into the SR. When phosphorylated, however, PLN loses its inhibitory action and allows SERCA to pump calcium back into the SR. In addition to SERCA to handle the high calcium concentration in the cytosol, there is also a sodium-calcium transporter (NCX). NCX utilizes the high concentration gradient of sodium to exchange for calcium anions, thereby clearing out remaining calcium. Additionally the mitochondria participate in calcium buffering as well through the mitochondrial uniporter (MCU). In the murine heart, 99% of calcium is cleared through SERCA, >1% is cleared through the NCX and a fraction of a percent through the MCU. Calcium release and uptake can be modified through post translational modifications, namely phosphorylation. This is often the case in a beta-adrenergic response where increased heart rate is required. Protein Kinase A (PKA) is the primary kinase associated with beta-adrenergic response. It can phosphorylate both the RYR and PLN, increasing the rates of calcium release and uptake. This allows the heart to fine tune contractile performance in order to meet increased demand.
1.4.3 The Extracellular Matrix

The extracellular matrix (ECM) is the support structure of the heart as well as the means through which nutrients and signals are communicated to the cardiomyocytes and resident fibroblasts. Normally it is not discussed in relation to cardiac function but the ECM is being recognized as an important contributor to heart function and health. Historically, the ECM has been considered simply the static mechanical support component of the heart and was not extensively studied. Recently, however, it has been recognized to be a dynamic, and responsive component of the heart. The primary composition of the ECM is collagen I and collagen III (col-I and col-III respectively); however, numerous other proteins are also active in the ECM such as proteoglycans, elastin, fibronectin, and laminin. Collagen is synthesized as a polypeptide chain inside the cells. In cardiac muscle this is done by the fibroblasts. These polypeptide chains have the conformation of a left-handed helix, three polypeptide chains twist together forming a right-handed triple helix. This is achieved by enzymes known as collagen peptidases which remove the ends of the procollagen strand. After the removal of the peptide ends pro-collagen becomes known as tropocollagen. Tropocollagen forms triple helical structures with other tropocollagens forming a fibril. These fibrils collectively form the collagen fibers which provide support for the heart and allow normal function. Increased collagen deposition often begins as a reparative or compensatory process in the heart in order to preserve cardiac function. This increased collagen deposition eventually becomes maladaptive and leads to a condition called fibrosis, ultimately reducing cardiac function. In this way, the ECM can provide
functional support to the heart but aberrant ECM remodeling can decrease heart function; therefore, the ECM represents a dynamic system directly contributing to cardiac function.

1.4.4. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a group of enzymes in the metzincin superfamily, enzymes that have a zinc active site, that specialize in degrading components of the ECM. MMPs have very conserved structure, consisting of a signaling domain, a propeptide region, the catalytic domain, hinge region, and hemopexin-like domain. As the MMPs are synthesized intracellularly and move into the extracellular space, the signaling domain determines where the MMP will locate. The propeptide domain is responsible for covering the zinc active site and preventing inappropriate enzymatic activity. MMPs need to have this domain removed before they can perform any enzymatic functions. The hinge region is a flexible region, approximately 75 amino acids long, that bends in order to place the propeptide domain over the zinc active site. The zinc active site is contained within the catalytic domain, which is largely responsible for determining MMP specificity. The hemopexin like domain is thought to help bind the tissue inhibitors of metalloproteinases (TIMPs) which are necessary for carefully regulating the activity of MMPs. There are additional modifications which warrant discussion. MMP-2 and -9 belong to a subgroup called gelatinases. The gelatinases have increased specificity for Col-I and Col-III, because of fibronectin repeats in the catalytic domain which are unique to that subfamily. Additionally, there are some MMPs which
are bound to the membrane, referred to as membrane type-matrix metalloproteinases (MT-MMPS). These MT-MMPS have an additional anchoring domain at the end of the hemopexin like domain that anchors them to the cellular membrane. MMPs are a relatively recently discovered class of enzymes, first being characterized in 1962. The first described MMP was MMP-1 which was found to be essential in the normal development of tadpole tail regression during metamorphosis. Since then, almost thirty unique MMPs have been characterized in vertebrates with the most recent, MMP-28, being characterized in 2001 (58). MMPs are synthesized as zymogens, enzymes in an inactive form, and are activated in the extracellular space through removal of the inhibitory domain. Once the inhibitory domain is removed, the active site, containing a zinc residue, is exposed allowing enzymatic activity. The zinc active site breaks apart extracellular proteins through hydrolysis. Although MMP activity is upregulated in certain disease states, MMPs are responsible for normal ECM maintaince, especially in the heart. Due to the on demand constant pumping of the heart the support structure, the ECM, becomes worn out and requires constant removal of proteins as well as synthesis of new ECM components. MMPs primarily regulate this cycle of removal and renewal through their enzymatic action. Degradation of proteins also causes the stimulation of growth factor release, such as TGF-β stimulating the synthesis of new ECM proteins. During a disease state, heart failure as an example, the levels and activity of MMP become markedly increased and contribute to the disease pathology. The role of MMPs in disease will be explored further.
1.4.4.1 Gelatinases (MMP-2 and MMP-9)

MMP-2 and -9 belong to their own family, referred to as gelatinases. As mentioned previously, these two MMPs have fibronectin repeats which contributes to their specificity. MMP-9 is often considered the basal MMP in the heart and is responsible for the routine ECM maintenance; however, it can become highly activated during heart failure. MMP-9 has been shown to be increased as early as the day after myocardial infarction (MI) and been shown through transgenic models to be critical in the remodeling process. Knockout MMP-9 mice when, subjected to an MI, show decreased chamber cavitation up to 15 days post MI (29). This was largely attributed to the early response MMP-9 plays in the rupture phase of the MI. This MMP-9 KO showed reduced leukocyte infiltration immediately following MI, indicating also that immune infiltration plays a role in MI pathology. MMP-2, the other gelatinase, has also been implicated in the pathology of heart failure. While MMP-9 appears to be responsible for the early stages of heart failure, MMP-2 is associated with the chronic phase of heart failure. As heart failure progresses from the initial insult to the dilation stage, MMP-2 increases. Increase in the serum level of MMP-2 in heart failure patients is associated with an increased risk of mortality. These findings were echoed in mouse MMP-2 KO studies of heart failure, which was shown to have reduced remodeling and increased survival times (46, 86, 118). MMP-2 and -9 play an important role in the onset and progression of heart failure but there are other MMPs that warrant further discussion.
1.4.4.2 Stromelysin (MMP-3)

MMP-3, unlike MMP-2 and -9, belongs to the stromelysin family along with MMP-10 and -11. MMP-3 lacks the fibronectin repeats of MMP-2 and -9, which confer collagen I and III specificity. MMP-3 substrates are more commonly fibronectin, elastin, and proteoglycans, among a few others in the heart (143). While MMP-3, like MMP-2 and -9, has been shown to be upregulated in the failing heart and showed a positive correlation between serum concentrations and complications, it is more well known for its role in regulating other MMPs. MMP-3 can act as a proteolytic factor on MMP-1, -7, and -9. It can actively break the bond that causes the inhibitory domain to cover the enzymatic active site (102, 138). Additionally, MMP-3 has recently been shown to translocate to the nucleus, something previously unseen in matrix metalloproteinases. It has been shown to act on connective tissue growth factor (CCN2/CTGF) (30). This transcription factor is highly involved in wound healing, proliferation, and several fibrotic remodeling pathways. Its regulation by MMP-3 may contribute directly to the advancement of the fibrotic phenotype, although more work needs to be done. Unlike MMP-2 and -9, MMP-3 has not been studied extensively in the context of the failing heart; however, its ability to regulate other MMPs along with possibly affecting the fibrotic response warrants further investigation.
1.4.4.3 Membrane Type-1 MMP (MMP14)

MMP-14, unlike most other MMPs, is membrane bound to an anchoring domain located on the C-terminus. MMP-14, like MMP-3, has been shown to be more involved in MMP activation rather than direct enzymatic action. MMP-14, along with TIMP-2, serves to activate MMP-2 through removal of the inhibitor domain (96). In heart failure, due to myocardial infarction, MMP-14 has been shown to be a late stage protein. It is only significantly increased approximately sixteen weeks after the cardiac event. This may contribute to remodeling after the initial injury where the highly inducible MMPs, such as MMP-9, are markedly increased. Like MMP-3, the exact role of MMP-14 in heart failure is unknown, but due to its interaction with other MMPs, it has become recognized as an important contributor to heart failure pathology.

1.4.5. Heart Failure

Heart failure is a general condition in which the heart is no longer able to adequately supply oxygenated blood to the body. Heart failure can have numerous causes such as high blood pressure, myocardial infarction, ischemic heart disease, and infection. Heart failure can be divided into left sided and right sided heart failure. Left sided heart failure is more common and will be the only type of heart failure discussed. Left sided heart failure can present with two different pathologies; systolic and diastolic heart failure. Systolic heart failure is heart failure where ejection fraction is compromised, usually due
to structural remodeling of the heart. Diastolic heart failure is heart failure where ejection fraction is preserved and is caused by disruption in the relaxation of the heart. Systolic compared to diastolic heart failure can largely be thought of as a problem of heart contraction as opposed to problems filling with blood. Heart failure, with a few exceptions, is an irreversible process that usually worsens over time. Complications of heart failure include fatigue, exercise intolerance, and depression. These complications become aggravated as heart failure progresses, reducing the quality of physical and mental well-being, causing overall decreased quality of life. Currently, more than 5 million patients in the United States suffer from heart failure, with 555,000 new cases occurring every year. It is the number one cause of hospitalization in people older than 65 in the United States, and, as the standard of living increases worldwide, the hospitalization rate for heart failure increases as well. Heart failure also represents a significant economical drain in addition to the personal and interpersonal complications (85, 92). In the United States heart failure will cost approximately 35 billion dollars due to hospitalizations. In the United Kingdom, heart failure costs will amount to approximately 2% of the National Health Service budget. While there are treatments for heart failure, they are often palliative and aimed at improving quality of life as curing heart failure is not possible without a whole heart transplant. Because of this, prevention of cardiac diseases are focused at changeable life style habits such as weight management and exercise. Heart failure, as a consequence of extrinsic factors such as infection, chemotherapeutics, and cancer, represents a special need. For these cases therapeutics need to be identified that can be safely administered to prevent the onset of heart failure.
Even if the underlying cause is resolved heart failure will persist, reducing the quality and expectancy of life in addition to the economic burden. Understanding the causes of these extra-personal factors to develop pre-emptive therapies will be crucial to preserve cardiac function and maintain quality of life once the underlying cause is corrected.

1.5. Tumor Induced Cardiac Dysfunction

Tumor induced cardiovascular dysfunction is new in terms of the field of cachexia with only a handful of papers being published in the last few years. There is currently no agreement in the mechanisms concerning cardiac dysfunction since cardiac muscle does not seem to respond in the same way as skeletal muscle. What is definitely known is that multiple models of cancer induced cachexia show reduced fractional shortening indicative of heart failure. There are significant discrepancies between the two studied models of cardiac dysfunction. In the colon-26 adenocarcinoma model there is significant decrease in the systolic wall thickness but no decrease in the diastolic wall thickness (131, 132, 150). This indicates that there is not a canonical dilated cardiomyopathy where the chamber would be expanded in both the systolic and diastolic measures which would be expected in atrophic remodeling. A model of rat liver cancer induced cachexia, however, shows a more expected dilated left ventricle phenotype (127). This indicates that there are differences depending on the type of cancer studied. There is a significant amount of work necessary to understand the mechanisms of tumor induced
cardiovascular dysfunction. The general mechanisms of cachexia will be described further and when applicable it will be related to heart failure.

1.6. The Colon-26 Adenocarcinoma Model of Cachexia

The colon-26 (c26) adenocarcinoma model of cancer cachexia has become a widely used model of cancer induced cachexia. Originally the tumor was cause by the administration of N-nitroso-N-methylurethane to induce tumor development. The tumor was isolated and immortalized into a cell suspension becoming the c26 commonly used today. The c26 cells, when injected subcutaneously, form a solid mass tumor that does not metastasize and is capable of inducing cachexia. This cachexia is rapidly induced over the course of 21 days with tumor burdened mice losing over 20% of their body mass during this time period. There is also significant wasting of the skeletal muscles and almost complete loss of adipose tissue. The c26 model cachexia is primarily driven through the action of IL-6, causing markedly increase levels of the cytokine found in the serum in addition to cause tissue specific IL-6 production (10). The c26 model has proven useful in detailing the mechanisms of cachexia which will be described below.
1.6 Mechanisms of Cachexia

1.6.1 The RAAS Pathway

The Renin-Angiotensin-System (RAS) pathway is a hormone system that plays a role in many physiological processes, but especially regulating fluid balance and blood pressure. The RAS pathway is triggered by a reduction of blood pressure, which results in the release of renin from the kidneys. This then acts on angiotensinogen in the liver, converting it to angiotensin I. Circulation to the lungs exposes angiotensin I to angiotensin converting enzyme (ACE) which converts angiotensin I to angiotensin II. While angiotensin I has effects of its own angiotensin II has more systemic effects including increasing sympathetic activity, vasoconstriction, and fluid retention. One of the effects of angiotensin II is stimulation of hypertrophic growth signals through the Type-1 Angiotensin receptor (AT1) found in both cardiac and skeletal muscle. In skeletal muscle extracellular angiotensin II can cause local angiotensin II production. The hypertrophic response of the heart in the early stages of heart failure is mediated by angiotensin II. Angiotensin II has also been implicated as the causative agent of wasting in cancer cachexia, sarcopenia, and other wasting diseases (152, 155). Angiotensin II has been shown to directly induce protein catabolism in both in vitro and in vivo models. An experiment in which rats were directly infused with angiotensin II, showed decreased food intake, as well as marked weight loss. However, because of the decreased food intake the results could not be linked to a pressor effect. This study was repeated with
pair-fed controls, showing that angiotensin II not only cause decreased food intake but protein catabolism as well, demonstrating a significant effect (113, 124). The mechanism behind angiotensin II mediated catabolism warranted further investigation. In separate experiments, it was found that angiotensin II catabolic effects were mediated by the disruption of insulin like growth factor-1 (IGF-1). IGF-1 in a normal setting causes angiotensin II to elicit hypertrophic signals. Angiotensin II mediated disruption of IGF-1 caused atrophic signals and activation of the E3 ubiquitin ligases. This was further confirmed by studies showing muscle specific overexpression of IGF-1 prevented the catabolic effects of angiotensin II (124). There is also evidence that inhibition of 5’ AMP Activated Protein Kinase (AMPK) activity by angiotensin II disrupts normal energy balances in skeletal muscle, inhibiting anabolism and promoting catabolism. Studies have found that circulating levels of angiotensin II correlate with increased inflammatory hormone and cytokine levels, such as tumor necrosis factor alpha, serum amyloid alpha, and interleukin-6. Angiotensin II has been shown to elicit multiple catabolic effects and may represent one of the primary initiators of cachexia. Treatment with ACE inhibitors, such as perindopril, have been shown to reduce the wasting and ubiquitin ligase activation in experimental models of cachexia (94). Additionally, direct inhibition of angiotensin II receptor binding, through the use of antiogensin receptor blocker (ARB), losartan, has shown improvements in body mass preservation in experimental models of cachexia (129).

Aldosterone, a part of the RAS system, is a steroid hormone, unlike angiotensin which is a peptide hormone. Aldosterone is a mineralcorticoid that functions to restore
salt and water balances to maintain normal function. The role of aldosertone in the wasting of cachexia is poorly described. Aldosterone has also been linked to the neurohormonal cascade contributing to inflammation and generation of reactive oxygen species. In a rat model of liver cancer, that induced cachexia, the antimineralcorticoid, Spironolactone, was shown to improve body weight as well as cardiac function. Another finding was that treatment with imadipril, an ACE inhibitor, showed no improvement in metabolic dysfunction or overall cardiac function (127). Additionally, it was found that aldosterone was elevated in human cancer patients with or without cachexia. This indicates that, even in the absence of angiotensin II mediated catabolism, the RAS pathway is still active in wasting through the action of aldosterone.

1.6.2 Interleukin-6

Interleukin 6 (IL-6) is an important inflammatory cytokine in several kinds of disease processes. IL-6 is named because originally the cytokine was discovered to be secreted from leukocytes, members of the innate immune system commonly called white blood cells. Since then it has been discovered in a number of cells and is an important mediator in the acute phase response. Despite its role as an inflammatory and negative cytokine, IL-6 has been shown to be crucial for muscle growth and hypertrophy. During the hypertrophic response, the presence of IL-6 is transient and not a high magnitude. Administration of systemic IL-6 or through electroporation that would result in superphysiological amounts of IL-6 have been shown to cause muscle atrophy (15). In
cancer cachexia, there is not only a marked increase in the amount of IL-6 but also the duration, leading to its investigation into the involvement during cachexia. IL-6 in cancer cachexia has been shown to either be a predictor or a result of weight loss, with small lung cancer patients showing increased amounts of IL-6 before weight loss and then a nine-fold increase at 5% weight loss (115). In a separate study, comparing 28 patients with different tumors, IL-6 was the only cytokine that was found to be increased in all the patients while TNFα was found to be increased in only one patient. The same study found a positive correlation between the amounts of IL-6 and the mortality of the patients, patients expressing higher amounts of IL-6 died earlier than those expressing a lower amount (28). Several animal models of neutralizing IL-6 during the course of cancer cachexia have also been utilized to further demonstrate IL-6 role in cachexia. The c26 mouse model has been shown to be largely dependent on IL-6 for the muscle atrophy response. When IL-6 neutralizing antibody was given to c26 tumor bearing mice the cachectic phenotype was not seen. When c26 tumor bearing mice were given a TNFα antibody, there was no prevention of the cachectic phenotype, reinforcing the role of IL-6 in cachexia (130). A common critique of the c26 mouse model is its aggressiveness and tumor burden, with the mice becoming moribund after approximately three weeks and the tumor reaching masses approximately 10% of the mouse body mass. Because of this, other models were used in determining the relation of IL-6 to cancer cachexia, namely the ApcMin mouse. The ApcMin mouse has a genetic defect that causes the growth of polyps throughout the intestine and colon, causing cachexia over a much longer time period, approximately 6 months, and is considered to represent the human cachectic
condition better. Like the c26 mouse, the ApcMin model does not show an induction of 
TNFα over the course of cachexia and that the amount of serum IL-6 was correlated to 
the degree of cachexia. In order to determine the contribution of IL-6 in this model, an 
IL-6 knockout with the ApcMin background was generated. This ApcMin IL-6 -/- mouse 
had improved body mass and muscle mass, comparable to controls, and did not present 
with the cachectic phenotype (11, 146). The ApcMin IL-6 -/- still did have polyp 
formation in the intestine and colon, indicating that the tumor burden alone was not 

enough to cause cachexia and required IL-6. While these studies have shown the 
importance of IL-6, the mechanisms of IL-6 atrophy induction warranted further 
investigation.

The mechanisms behind IL-6 induced atrophy are still being fully described; 
however, studies have elucidated some possible mechanisms. IL-6, as a ligand interacting 
with its receptor, gp130, activates the JAK-STAT pathway. The binding of gp130 
activates Janus Kinase (JAK) which then acts on signal transducers and activators of 
transcription (STAT) through phosphorylation. Once STAT is phosphorylated, it can 
dimerize and translocate to the nuclease causing transcriptional changes. One of the 
changes thought to be linked to cachexia is the involvement of STAT3 in reducing the 
expression of IGF1 receptor. The balance between IGF1 receptor and circulating IGF1 is 
an important regulator in maintaining muscle mass. With the receptor expression being 
decreased, IGF1 interaction is no longer able to maintain anabolic and survival signals, 
leading to protein synthesis suppression. In order to prove that STAT3 was acting 
downstream of IL-6 and responsible for the cachectic phenotype a dominant negative
STAT3 was used in the c26 mouse model of cachexia (15). This dnSTAT3 showed not only increased resistance to the cachectic phenotype but increased fiber size when compared to control mice. IL-6 mediated cancer cachexia is shown to occur through the activation of the JAK-STAT pathway and not through the E3 ligase pathway. The role if IL-6 is still controversial.

### 1.6.3 Tumor Necrosis Factor α

Tumor Necrosis Factor α (TNFα) is another cytokine that is involved in inflammation and one of the cytokines, along with IL-6, that make up the acute phase reaction. The role of TNFα in cancer cachexia is not as well defined as IL-6 but it has been shown to be a possible therapeutic in the treatment of cachexia. While TNFα can be produced by multiple cell types, it is most commonly expressed by immune cells, especially activated macrophages. In experimental models of cancer cachexia receptors for TNFα, TNFR1 and TNFR2, are increased at both the transcriptional and protein level in skeletal muscle and adipose tissue. High TNFα serum levels, however, seems to have differential effects on adipose tissue compared to skeletal muscle. There is evidence that circulating TNFα increases TNFα expression in adipose tissue, while TNFα expression is decreased in skeletal muscle (32). In adipose tissue, TNFα has been shown to induce lipid depletion in white adipose tissue (WAT) through transcriptional suppression or the active upregulation of lipolytic factors. TNFα has been shown to suppress perilipin (PLIN), which protects lipids from naturally occurring lipases and takes part in the mobilization
and storage in adipose tissue. TNFα suppresses PLIN function through the MAPK pathway. TNFα mediated activation of MAPK causes PLIN to become hyperphosphorylated. When PLIN is hyperphosphorylated it is no longer able to perform its protective function and lipids are broken down by lipases. Other studies performed in cultured adipocytes have shown that TNFα also decreases PLIN protein and gene expression. This decrease in PLIN is paralleled directly by increased basal lipolysis (154). These studies indicate that TNFα may be exerting multiple effects over the short term, such as MAPK activation, or longer term effects such as reduction in PLIN expression. In skeletal muscle, TNFα also causes wasting but the mechanisms are different than in adipose tissue. As mentioned previously, circulating TNFα in serum reduces TNFα expression in skeletal muscle, implicating that wasting is caused by secondary effects. This has been supported by in vitro experiments, demonstrating that direct TNFα administration to incubated muscles was insufficient to increase proteolysis. TNFα is thought to indirectly induce muscle proteolysis through increase in the MAPK pathway which in turn upregulates Atrogin-1/MuRF-1, both E3 ubiquitin ligases. TNFα has also been shown to induce muscle wasting through NF-κB mediated activation of the E3 ligases. In incubated muscle studies, the amount of TNFα concentration correlated with the decrease in IκB, which is the inhibitory protein of NF-κB. Decreases in IκB free NF-κB to move into the nucleus and elicit transcriptional effects. This mechanism is not consistent with every model, however, indicating that TNFα may be eliciting skeletal muscle wasting through the E3 ligases but via two different pathways, MAPK or NF-κB. Despite experimental evidence of TNFα mediated muscle wasting in animal models,
human studies demonstrating TNFα mediated wasting are not consistent (75). Some studies demonstrate an inverse correlation with TNFα presence in serum and body weight and mass index. However, other studies have demonstrated significant body weight loss despite undetectable levels of TNFα in serum. TNFα expression by activated macrophages is transient by nature, complicating the interpretation of human studies involving TNFα. There is also a multitude of cytokine signaling networks that become activated in the sustained inflammation associated with cachexia, making individual study of cytokines problematic. TNFα expression may be related to tumor type, size, and location, indicating the extent of heterogeneity between tumor wasting mechanisms and warranting further study in human and animal models.

1.6.4 Ubiquitin Proteasome Pathway

The ubiquitin proteasome system is a system found in several organisms including archae, eukaryotes and some bacteria. Its function is to degrade damaged, old, and misfolded proteins, and achieves this by breaking the peptide bonds through hydrolysis. The proteasome complex is named after its sedimentation coefficient, or Svedberg unit. In mammals, the 26S proteasome is exclusively used which will be discussed further. The proteasome complex is composed of three proteins; a 20S core and two flanking 19S regulatory caps. These form a three dimensional structure similar to an open ended barrel. Proteins marked for degradation through ubiquination, pass through this barrel, which is regulated by the 19S regulatory unit. As the protein passes through the barrel, its peptide
bonds are broken through hydrolysis and the fragmented peptide units pass through the other end of the barrel. In order to prevent inappropriate protein degradation the protein must be marked for breakdown, this is done through ubiquination. Ubiquination is a process where a protein has ubiquitin, a protein itself, attached to it. Typically the ubiquination is carried out multiple times causing the target protein to have a long chain of ubiquitins on it. At this point, it is recognized for proteolysis by the proteasome. The process of ubiquination itself is also regulated, involving a three-step process in order to ubiquinate a protein. The first classes of proteins in the ubiquitin process are called ubiquitin activating protein, or more simply E1. These proteins have a ubiquitin covalently attached to a sulfhydryl group through an ATP dependent mechanism. This ubiquitin is then passed to the ubiquitin conjugating enzymes, commonly called E2. From there, the E2 forms a complex with the ubiquitin ligase, which is referred to as the E3 ligases, which form a complex with the protein to be targeted. The E3 ligases confer specificity to the E2 conjugating proteins. Once the protein is recognized, the thioester ubiquitin bond is broken and the ubiquitin transferred to the targeted protein. Since E3 ligases are the proteins the confer specificity to the ubiquitin system, they have received attention in the field of cancer cachexia. There are two E3 ligases that stand out as being markers of cachexia which will be discussed further, Muscle RING Finger Protein-1 (MuRF-1) and Muscle Atrophy F-box (MAFbx).
1.6.4.1 MuRF-1

MuRF-1 is an E3 ubiquitin ligase that has been associated with the degree of wasting in cachexia, along with other wasting diseases. MuRF-1 belongs to the TRIM family of proteins and has a conserved structure associated with it, a tripartite motif and zinc based RING finger. The RING finger confers specificity to MuRF-1, and it has been shown to localize along the sarcomere. Although cardiac function in the setting of cachexia has not been thoroughly investigated the function of MuRF-1 in cardiac function can be inferred from other studies. MuRF-1, as mentioned previously, locates to the sarcomere and in the heart is especially relevant in the degradation of myosin heavy chain and troponin I. This degradation directly affects cardiac function resulting in decreased contractile performance as well as the times of contraction in isolated cardiomyocytes. In a murine model of heart failure, MuRF-1/MAFbx siRNA administration to mice corrected contractile performance and caused significant improvement (2). Transgenic over-expression of MuRF-1 in the murine heart also causes progressive ventricular thinning with increased sensitivity to the trans-aortic constriction model of heart failure. Despite the implications that MuRF-1 has deleterious cardiac effects, MuRF-1 has also been shown to be cardioprotective in some instances (147). A transgenic over-expression model, when subjected to ligation of the left anterior descending coronary artery, showed a protective effect through proteasome-dependent degradation of c-Jun. Targeted destruction of c-Jun prevented the increase of apoptotic markers in the ischemic reperfusion model, protecting cardiac function. MuRF-1 has also been shown to regulate
reactive oxygen species (ROS) in mitochondria, offering protection independent of anti-apoptotic signals in a similar model of ischemia reperfusion injury (74). The role of MuRF-1 in the cardiac system is multi-faceted and may be dependent on other factors that further regulate its activity.

1.6.4.2 MAFbx

MAFbx, also called Atrogin-1, is another E3 ubiquitin ligase; however, it differs from MuRF-1 in that it is not a member of the TRIM family. MAFbx is an F-box protein that forms a complex with Skp1 and Cullin, collectively called the SCF complex. MAFbx is what confers the substrate specificity of the SCF complex, marking proteins for ubiquination through a phosphorylation-dependent reaction. Although MAFbx can ubiquinate proteins and mark them for degradation, it more commonly inhibits protein synthesis through inhibition of upstream signaling. In the progression of heart failure, MAFbx typically is expressed at a low level during the hypertrophic response in heart failure. As the hypetrophic response becomes insufficient and dilation occurs, MAFbx expression increases (70). This is seen in transgenic mice over expressing MAFbx. At baseline there is no functional decrease as determined by echocardiography. Two weeks past baseline, however, echocardiography showed a decrease in the posterior wall along with expansion of the left ventricular chambers. While MAFbx marks protein for degradation through ubiquination, it more commonly is involved in the suppression of protein synthesis. During heart failure, MAFbx inhibits AKT activity, blocking
hypertrophic responses and allowing FOXO translocation into the nucleus (123). This begins a cycle of blunted hypertrophic response and increasing MuRF-1 expression, leading to degradation of sarcomere proteins.

1.6.5. Neuroinflammation

While skeletal muscle has primarily been the focus of studies in cachexia, recently there have been recent discoveries concerning the role of the brain in cachexia development. Pro-inflammatory cytokines that are up regulated during cancer cachexia in the c26 mouse have been found to increase inflammatory expression in part of the brain such as the hippocampus and cerebral cortex (101). Increases in the inflammatory cytokines in the brain have been associated with neurological changes, such as cognitive impairment and depression. Studies in different models of cancer cachexia have shown significant decreases in behavioral measures, such as voluntary wheel running and grip strength (93, 94). While decreases in grip strength were once thought to be a result of muscle weakness it has become recognized that it can be a result of a depressive state. This depression can further influence a lack of pleasure seeking, or anhedonia, which includes eating and drinking, along with locomotion. The molecular mechanisms for these effects are still under investigation but there are some prospective pathways that have been identified. The inflammatory cytokines could activate the innate immune system located in the brain, causing the activation of astrocytes (91). While this is normally transient and in response to infection, in the cachectic model this astrocyte
activation is sustained and contributes to the depressive phenotype by increasing local inflammation. Aside from the depressive phenotype, there is indication that there is depression of appetite. This is currently thought to be the result of inflammation activating the NOS pathway which has been shown to reduce the activity of Neuropeptide Y and increase the amount of Malonyl CoA, contributing to anorexia through activation of AMPK (111).

Although in studies of cancer cachexia there is no direct link between cardiac dysfunction and the depressive phenotype, inferences can be drawn from other depressive models. Intense depression can cause temporary heart failure known as takotsubo cardiomyopathy, colloquially known as broken heart syndrome. During this state the heart, when observed using echocardiography, shows a phenotype closely resembling that of a myocardial infarction, with thinning left ventricular wall and depressed function. One of the causes is thought to be the release of stress hormones such as epinephrine (106). The levels of epinephrine have been shown to be raised in a rat model of liver cancer cachexia. There have been no reports of epinephrine levels in other models of cachexia, however, the connection between epinephrine, depression, and cardiac function warrants further investigation.
1.7. Overall Goals and Significance

Currently, the relation of cancer cachexia and its impact on cardiac function is poorly understood. Studies are primarily still in the descriptive stages of determining the cardiac phenotype when applicable. The only information for cardiac function in cachectic models is the c26 mouse model and the AH-130 Yoshida rat model. While there has been a study concerning the heart in the ApcMin mouse, this study had no relevant functional data and only described the effect of tumor burden on the mTOR pathway in the heart. In the c26 model, the cardiac dysfunction associated with tumor burden has been shown to be ameliorated with the administration of losartan, an Angiotensin II receptor blocker, in addition to recovery of body mass. These findings, however, were not supported by the rat model of cachexia where ARBs were found to have no effect but the antimineralcorticoid, Aldosterone, improved cardiac function. While not surprising, these results indicate that there can be significantly different mechanisms by which cardiac dysfunction develops, based on the type of model. What has been confirmed is that there is significant fibrotic remodeling in the hearts of human patients who died as a direct result of cachexia. The cardiac dysfunction present is not solely limited to experimental models.

There could be significant healthcare concerns associated with cachexia induced cardiac dysfunction in human patients. Even if the underlying cause is fixed and cachexia reversed successfully, in the event of left ventricular compromise the patient may be at high risk for the development of heart failure. Not only could this increased risk lead to a
higher mortality, heart failure itself can cause cachexia. This double insult may prove
disastrous to patients who survived the initial cachexia and tumor burden. If the effects of
cancer cachexias impact on the heart can be further elucidated, it could be possible to
start preemptive treatment preventing possible cardiac complications to patients with
cachexia. There are significant healthcare concerns associated with uncovering the
mechanism of tumor induced cardiac dysfunction.
Chapter 2: Altered Metalloproteinase Expression in Cardiac and Skeletal Muscle in Cancer Cachexia

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2.1 Introduction

Cancer is a rapidly growing health concern worldwide, with 25% of all deaths attributed to cancer in the US (120, 121). During disease progression, approximately 30-80% of cancer patients develop cachexia, a syndrome characterized by marked wasting of adipose tissue and skeletal muscle (27). Cachexia not only increases the likelihood of morbidity from cancer, but is also directly responsible for 20-40% of deaths in cachectic patients (135). Cachexia is a detrimental syndrome, negatively affecting patient quality of life and potential for recovery (34, 144). Understanding the molecular mechanisms of cancer cachexia could assist in creating effective therapies to improve survival and quality of life in these patients.

Increased serum levels of pro-inflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α, play pivotal roles in tumor-induced muscle wasting
These cytokines increase activity of the ubiquitin proteasome and autophagy systems, promoting muscle protein breakdown (19, 62, 81). Ubiquitin activity may play a role in early skeletal muscle wasting; however, the ubiquitin pathway of degradation does not completely explain cachexia-associated pathology. For example, studies in human cancer patients have not correlated increased ubiquitin mRNA levels with weight loss (133). Therefore, other degradation systems likely contribute to skeletal muscle wasting in cancer cachexia.

In addition to influencing skeletal muscle mass and function, tumor burden also promotes cardiac dysfunction. CD2F1 mice, injected with colon-26 (C26) adenocarcinoma cells (10), are a recent established model to study cancer cachexia. In this model, tumor development is directly correlated with reduced cardiac function as measured by ejection fraction (EF) and fractional shortening (FS) (132). Although in vivo cardiac function was reduced in C26 adenocarcinoma induced tumor cachexia, in vitro analyses of isolated cardiomyocyte did not indicate cardiac dysfunction on the cellular level (150), suggesting the involvement the extracellular matrix in the development of cardiac dysfunction. Heart samples from human cancer cachexia patients show evidence of myocardial fibrosis (127), potentially associated with matrix metalloproteinase (MMP)-mediated alterations in the extracellular matrix (ECM).

Matrix metalloproteinases degrade several components of the ECM, resulting in tissue remodeling processes including angiogenesis (73). In addition, MMPs play active roles in cell proliferation and migration. MMPs are secreted in an inactive form that requires the removal of an inhibitory domain for activation (96). Additionally, endogenous
inhibitors of MMPs (tissue inhibitors of metalloproteinases (TIMPs)) inhibit MMP activity by directly binding to the MMP active site. MMPs are secreted by tumors cells and this facilitates angiogenesis for tumor growth and survival, as well as metastasis (22). However, the involvement of MMP activity in muscle wasting or dysfunction in cancer cachexia remains unknown. The purpose of the present study was to investigate MMP and TIMP production in skeletal and cardiac muscle in a model of cancer cachexia. We show that tumor-bearing mice have increased MMP levels in both cardiac and skeletal muscle, compared to control mice which may contribute to altered heart and skeletal muscle function associated with cachexia. These findings will provide insight into the contribution of MMPs to skeletal muscle wasting and cardiac dysfunction in cancer cachexia.

2.2 Materials and Methods

2.2.1 Mice

Adult (10 weeks old) female CD2F1 (BALB/c x DBA/2 F1) mice, weighing 20-22g, were obtained from Charles River Laboratories. Mice were housed 1-3 per cage and maintained at 25°C under a 12 hour light/dark cycle with ad libitum access to water and standard rodent chow. All animal care and procedures were approved by the Ohio State University Institutional Animal Care and Use Committee.
2.2.2 Mouse Model of Tumor Growth

The C26 cell line was maintained in culture and prepared for injection as previously described (34). Mice were randomly selected to receive the cell injection subcutaneously between the scapulae with $5 \times 10^5$ cells in 0.2 ml of PBS, while control mice were injected with PBS alone. Tumor growth was usually palpable by day twelve post-injection and mice became moribund typically by day twenty-four post injection. In this study, mice were euthanized by day twenty-one post-injection. Mice were euthanized using a ketamine/xylazine (10/1 mg/ml, respectively) cocktail at a volume of 0.01 ml per gram of body weight (approximately 0.2 ml) and blood was withdrawn from the brachial artery. Heart, hind limb muscles, and diaphragm were dissected, weighed, snap-frozen in liquid nitrogen, and stored at -80°C until biochemical analyses.

2.2.3 Samples and Preparation

The free wall of the left ventricle, diaphragm, soleus and extensor digitorum longus (EDL) muscles of control and tumor-bearing mice were used for electrophoretic gel and Western blot analyses. The muscles were dissected and homogenized (model PRO200 homogenizer, PRO Scientific Inc., Monroe, CT) in sample buffer (14) (30 µl per/mg muscle) for 15 seconds. The homogenates were then centrifuged at 14,000 rcf for 4 minutes. Supernatants of the samples were collected and heated at 65°C for 2 minutes and maintained on ice for 5 minutes. Samples were maintained in a -40°C freezer until they were analyzed.
2.2.4 Gel Electrophoresis

Separating (12% acrylamide with a 200:1 cross-linking ratio) and stacking (4% acrylamide, 50:1 cross-linking) gels were prepared as described in a previous paper (12). Gels were run at a constant current (30 mA per gel) for 3 hours at 18\(^\circ\)C in Hoefer SE600 units (Hoefer Scientific, San Francisco, CA). Gels were stained with Coomassie blue(12). The staining was used to determine the loading amount which was determined by the amount of protein in the actin region. Images of the gels were obtained with a digital camera (Spot Insight Firewire, model 18.2, Diagnostic Instruments, Inc., Sterling Heights, MI). The gels were analyzed with Image J software (National Institutes of Health).

2.2.5 Western Blot Analyses

Gels for immunoblotting were run and proteins were transferred onto nitrocellulose membranes at a constant voltage (100 V) for one hour. Membranes were incubated with 1% bovine serum albumin (BSA), dissolved in Tris buffered saline with Tween 20 (TBST), for 1 hour at 25\(^{\circ}\)C or overnight at 4\(^{\circ}\)C. The membranes were incubated with primary antibodies for 2 hours at 25\(^{\circ}\)C and washed with TBST solution with gentle shaking three times for ten minutes per wash. Then, the membranes were incubated with secondary antibodies for one hour at 25\(^{\circ}\)C. The membranes were washed with TBST solution for 30 minutes (three ten-minute washes with gentle shaking). Color was developed using the BCIP/NBT color development substrate (S3771, Promega, Madison, WI). Finally, the membranes were air dried and digital images were captured as described above for gel imaging. Images were analyzed with Image J software. Primary antibodies were anti-
MMP2 clone 2C1 (1:100 diluted), anti-MMP3 clone F-1 (1:100), anti-MMP9 clone E-11 (1:200), anti-MMP14 clone C-9 (1:200), anti-TIMP1 clone H-150 (1:100) and anti-TIMP2 clone 3A4 (1:200). All primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas). Primary antibodies were diluted with 0.3% BSA in TBST solution. Secondary antibodies were anti-mouse IgG1 (S3721, Promega; diluted 1:10,000) and anti-rabbit IgG (S3731, Promega; diluted 1:10,000). Secondary antibodies were diluted with 1% BSA in TBST solution.

2.2.6 RNA Isolation and Real-time Quantitative PCR

RNA was isolated from the free wall of the left ventricle, EDL, soleus, and diaphragm using a TissueLyser system in Trizol Reagent (Sigma, St.Louis, MO). RNA was extracted using RNeasy spin column purification (Qiagen, Valencia, CA.). RNA was quantitated and 300-500 ng of RNA were reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). Quantitative PCR was performed using a three-step protocol on the CFX96 Real Time System (Bio-Rad), as described previously(150). Briefly, cDNA was amplified using real time qPCR and was normalized based on reference cDNA (GAPDH). Data was analyzed using the $2^{-(ΔΔCt)}$ algorithm (18).

2.2.7 Picrosirius-Red Quantitation

Hearts from control and tumor-bearing mice were fixed in paraformaldehyde before being embedded in paraffin. The paraffin blocks were cut in 10 μm sections and were mounted on glass slides and rehydrated. Following rehydration, slides were placed in 0.1%
Sirius Red and diluted in saturated picric acid for 90 minutes. After washing, the slides were dehydrated with graded alcohols before being cleared and then mounted in synthetic media (16).

For histological analyses, slides from the area below the papillary muscles were identified and images were taken of the septum and the posterior, free, and anterior walls at a magnification of 200 using a BX 41 microscope (Olympus, Hamburg, Germany). Quantitation and planimetric analyses were performed using Analysis (Olympus, Hamburg, Germany). The picrosirius-red (PSR) positive-stained area was quantitated as a percentage of the total myocardial area (26).

2.2.8 Hydroxyproline Assay

Serum from control and tumor-bearing mice was used to perform a hydroxyproline assay (Sigma Aldrich, St. Louis, MO). Briefly the samples were hydrolyzed with hydrochloric acid before being spun at 13,000 x g. The sample was then treated with Chloramine-T to oxidize hydroxyproline and then Perchloric Acid was added to remove Chloramine-T. The oxidized sample was incubated with DBMA to produce a photometric product at 560 nm. The results were acquired using a Bio-Tek Power Wave (Bio-Tek, Winooski, VT) plate reader and calculated using a standard curve of hydroxyproline provided with the assay kit.
2.2.9 Statistical Analyses

Values from six control and six tumor-bearing mice are reported as mean ± SEM. The two groups were compared using Student’s t-test, where p< 0.05 indicates statistical significance.

2.3 Results

2.3.1 Tumor burden increases MMPs and TIMPs mRNA expression in striated muscle

MMP and TIMP mRNA expression was determined in skeletal and cardiac muscles of tumor-bearing mice to test whether they are elevated in association with cancer cachexia (Fig 2.1). In the left ventricle, MMP-2,-3, and -14 were significantly increased in tumor-bearing, compared to control, mice (Fig 2.1A). The skeletal muscle response for tumor-bearing mice differed from that of cardiac muscle. In the diaphragm, MMP-2 and -9 were significantly increased (Fig 2.1B), while the EDL and soleus both showed significant increases in MMP-3 (Fig 2.1, C-D).

Certain striated muscles of tumor-bearing mice had increased levels of TIMP-1(Fig 2.1), compared to those from control mice. The left ventricle had significantly increased TIMP-1 expression, but TIMP-2 was unchanged (Fig 2.1A). In striated muscle the diaphragm had significantly increased expression of TIMP-1, but not TIMP-2, while the EDL and soleus showed no increases in either TIMP-1 or TIMP-2 (Fig 2.1, B-D).
Figure 2.1: Gene expression changes of MMPs and TIMPs in striated muscles of control and C26 tumor-bearing mice. Gene expression levels were determined using the Livak method with GAPDH serving as the internal control. * P < 0.05 was considered statistically significant.
2.3.2 MMP and TIMP protein levels are increased in tumor-bearing mice

In the left ventricle, protein levels of MMP-2, -3, -9, and -14 were significantly increased compared to control values (Fig 2A). The EDL, soleus, and diaphragm demonstrated similar changes in MMP protein levels. The diaphragm, EDL, and soleus had significantly increased expression of MMP-2, -3, and -14, without changes in MMP-9 (Fig 2, B-D). Protein levels of TIMPs were increased in tumor-bearing mice. The left ventricle had significantly increased TIMP-2, but not TIMP-1, protein levels (Fig 2A). The diaphragm, EDL, and soleus showed significant increases in TIMP-2, but not TIMP-1 in a manner similar to cardiac muscle (Fig 2.2, B-D). Representative immunoblots are shown in Figures 2.3 and 2.4.
Figure 2. 2: Quantitation of MMP and TIMP protein expression levels in striated muscles of control and C26 tumor-bearing mice. Protein levels were expressed as the ratio between the target protein (MMP or TIMP) and actin, the reference protein. * P < 0.05 was considered statistically significant.
Figure 2. 3: Representative immunoblots showing changes in MMP-2, -3, -9, and -14 in cardiac and skeletal muscles from control and tumor-bearing mice.
2.3.3 Hydroxyproline is increased in the serum of tumor-bearing mice

In order to assess increased collagen turnover we analyzed the serum of mice for the collagen by-product, hydroxyproline. There was a significant increase in the amount of hydroxyproline in the serum of tumor-bearing mice (Fig 2.5), indicating increased MMP activity and collagen turnover.
Figure 2.5: Quantitation of hydroxyproline in the serum of control and tumor-bearing mice. * P<0.05 was considered statistically significant.
2.3.4 Collagen deposition is increased in the left ventricle of tumor-bearing mice

In order to assess increased collagen deposition in the left ventricle, we analyzed Picrosirius-Red stained sections. There were significantly increased amounts of collagen deposition in the left ventricle of tumor-bearing mice compared to control mice (Fig. 2.6). Collagen deposition, as the percentage of the total area, was significantly increased in tumor bearing mice.
Figure 2.6: Collagen deposition in the left ventricle determined by Picrosirius Red staining (A) and quantitated as a percentage of the total area (B). * P<0.05 was considered statistically significant.
2.4 Discussion

In the current study, we found that tumor burden increased the mRNA expression and protein amount of MMPs and TIMPs in striated muscle. Up-regulation of the ubiquitin proteasome pathway has been established in cancer-induced cachexia (69), but other proteolytic or degradation pathways have not been extensively studied. This study is the first to demonstrate that cancer-induced cachexia in a mouse model is associated with increased expression of MMPs and TIMPs in skeletal and cardiac muscle. This increased expression of MMPs could contribute to the cardiovascular and skeletal muscle pathologies observed in the tumor-burdened mouse. The cardiac phenotype in the cachectic mouse model used in this study exhibits decreased *in vivo* function, but not decreased isolated cardiomyocyte contractility. The increased levels of MAFbx/MuRF-1 found in our model are expected to negatively affect cardiomyocyte contractility (2, 61). The action of MAFbx/MuRF-1 was demonstrated in a rat model of chronic heart failure where increases in MAFbx and MuRF-1 resulted in contractile dysfunction that was abrogated by treatment with MAFbx/MuRF-1 siRNA (2). However, we did not observe cardiomyocyte dysfunction 21 days following tumor cell injection (150). While there is an increase in MAFBx/MuRF gene and protein expression in the heart of cachectic mice (131, 150), the lack of changes in cardiomyocyte contractile properties indicates that the functional changes are not entirely due to the ubiquitin system.

Increases in MMPs have been implicated in heart failure and morbidity in heart failure patients. The gelatinases MMP-2 and -9 (13, 118) are most commonly associated with alterations of the ECM in heart failure. We observed significant upregulation in both
enzymes at the protein level. The collagenase MMP-9 was not increased at the RNA level but was increased at the protein level. The MMP-9 protein is often present at a basal level and activated post-transcriptionally during inflammatory states, possibly explaining the discrepancy in RNA level compared to the protein level. MMP-3 and MT1-MMP (MMP-14) have not been directly implicated in cardiac remodeling, but more often regulate the expression and activity of other MMPs (141). MMP-3 may regulate MMP-2 and -9 in heart failure models, while MMP-14 activates MMP-2. The increases in collagen deposition, determined by both PSR staining and hydroxylproline assay, demonstrate that MMPs are actively remodeling the ECM and increasing collagen deposition. This MMP activity and matrix remodeling is most likely responsible for decreases in wall thickness during contraction, contributing to decreased cardiac function.

Skeletal muscle loss has been an intense area of focus within the cachectic field due to the wasting present in both human cancer patients and in animal models of cancer. This wasting is due to activation of the ubiquitin proteasome system, but does not involve a reduction in the total number of muscle fibers or altered force generation per unit of cross-sectional area (93, 114); although others report a significant reduction of force/cross-sectional area in the murine C26 model (110) and in human patients (139). Skeletal muscles in some cachectic models are unable to generate new muscle cells due to the inability of satellite cells to mature into myoblasts (48). MMPs can contribute to the differentiation of satellite cells (103). In canine and murine models of muscular dystrophy, MMPs promote the release of satellite cells as well as the release of growth factors that stimulate proliferation (38, 63). There is evidence that MMP-2 and -9 activities contribute to the
differentiation of satellite cells in mdx mice. However, MMP-2 and -9 have been shown to degrade the extracellular matrix and to prevent muscle regeneration in inflammatory myopathies (63). The extent of MMP upregulation appears to play a role in their specific activity, a small upregulation can release satellite cells and permit differentiation while a more profound increase can disrupt the ECM and prevent differentiation. At the RNA level only MMP-3 is upregulated in the soleus and EDL, while at the protein level MMP-2, -3, -14 and TIMP-2 are significantly increased. It is possible that skeletal muscle has higher basal amounts of RNA, negating the need for a prolific gene response while maintaining a robust protein response. This aberrant increase in MMPs in the EDL and soleus in the cachectic mouse model may contribute to the inability of satellite cell proliferation by breaking down the surrounding matrix and disrupting normal cell survival signals transmitted through the matrix and between cells.

Diaphragm strips from tumor-bearing mice generate decreased peak specific force compared to normal mice (93, 110). Cachectic patients undergo respiratory complications due to failure of the diaphragm (110). The decreased peak specific force of the diaphragm in tumor bearing mice could be the mechanism by which human patients experience respiratory complications. Increased MMPs in the diaphragm could change the underlying architecture of the ECM contributing to changes in the physical and mechanical properties of the diaphragm. Additionally, while MMPs perform most of their proteolytic actions extracellularly, they have been shown to localize to sarcomeric proteins in the heart (3), and it is possible that they are able to localize to sarcomeric proteins in other muscle types. In a mouse model of mdx, there was decreased specific force of the diaphragm (49, 128). A
separate study showed that decreased force production could be ameliorated by the MMP inhibitor, Batimastat (65). However, Kumar et al. investigated several MMPs as well as A Disintegrin and Metalloproteinases (ADAMs) making contributions of specific MMPs in respiratory pathology difficult to evaluate. The roles of MMPs in diaphragm and respiratory dysfunction warrant further investigation.

In this study, we have demonstrated a significant increase of specific MMPs in cardiac and skeletal muscles at the RNA and protein level. Currently, the only other studies reporting changes in MMPs in both cardiac and skeletal muscle were done in muscular dystrophy models. In the mdx model, increases in MMP-2 and -9 correlated with cardiomyopathy in mice. This cardiac dysfunction is also coupled with skeletal muscle dysfunction. As mentioned above treatment of mdx mice with Batimastat, a broad MMP inhibitor, improved skeletal muscle function and significantly reduced fibrosis (65). MMP-2 and -9 have been most heavily studied with regard to both cardiac and skeletal muscle; however, it is known that MMP-3 can regulate the activity of other MMPs and MMP-14 can activate MMP-2. These MMP-induced MMP expression and activity changes can account for some of the differences in gene expression, as compared to protein differences. MMPs could cleave other substrates leading to a pro-transcription rate while not altering the amount of gene expressed. While we found increased protein expression of TIMP-2, which is capable of inhibiting all MMPs, from our histology and serum assays we can infer that TIMP-2 is no longer providing adequate MMP inhibition. TIMPs, while having a high affinity for MMPs, can bind the active site in a one-to-one ratio, and this can significantly limit their inhibitory activity in cases of marked increases in MMPs.
Further research is needed to elucidate the role of MMPs in the cardiac and skeletal muscle dysfunction in cancer cachexia. Determining to what extent MMPs contribute to the pathology will potentially lead to the development of effective treatments for cachectic patients, especially with respect to cardiac complications due to tumor burden.
Chapter 3: Minocycline Attenuates Cardiac Dysfunction in Tumor-Burdened Mice

3.1 Introduction

Cancer is a pervasive disease that was the leading cause of deaths world-wide in 2014 (120), and is predicted to be the leading cause of death in 2015 (55, 85). Of those who develop cancer, approximately half will develop cachexia. Cachexia is a wasting syndrome, characterized by marked loss of adipose tissue and skeletal muscle mass (27). Cancer-induced cachexia will be the primary cause of death in a quarter of those affected (133). While primarily thought of as a syndrome affecting adipose tissue and skeletal muscle, recent investigations have determined that cancer-induced cachexia also negatively affects heart function. In animal models of cancer-induced cachexia, there is a significant decrease in fractional shortening (FS) via echocardiography, consistent with heart failure (150). While no human studies have determined the cardiac functional alterations caused by cancer cachexia, there is histological evidence from patients who died from cancer cachexia that there was marked fibrotic remodeling (127). Remodeling in heart failure is an accumulation of multiple factors, but is strongly dependent on the activity of matrix metalloproteinases (57, 76, 125).

Matrix metalloproteinases (MMPs) are zinc-peptidases that alter the structure of the extracellular matrix (ECM) by hydrolyzing the peptide bonds holding proteins together (73, 96). MMPs are a normal component of cardiac homeostasis but
uncontrolled MMP activity leads to detrimental remodeling that can negatively affect cardiac function (118). MMPs are endogenously controlled by Tissue Inhibitors of Metalloproteinase (TIMPs) (96); however, previous research in the c26 mouse model of cancer cachexia showed an insufficient TIMP response to compensate for increased MMPs. This insufficient response leads to MMP-mediated collagen deposition in the heart, presenting as fibrosis, which can compromise heart structure and negatively impact function. While MMP levels were increased in the c26 cachectic mouse model (24), the degree in which they participate in remodeling remains unknown. In order to determine how increased MMP activity could lead to altered function, we utilized a known inhibitor of MMPs, minocycline.

Minocycline is a semisynthetic tetracycline derivative first synthesized in 1967 by Ledele Laboratories. It quickly gained FDA approval in 1970 as a broad spectrum antibiotic and is still used today for both its antibacterial effects and recently discovered neuroprotective effects. It has been used as an MMP inhibitor in diseases primarily caused through MMP activity in both animal and human studies (54, 79, 122). Because of its current status as an FDA approved drug and its well documented use and tolerability in humans, minocycline represents an attractive treatment strategy for cancer-induced cardiac dysfunction in humans.

In the present study, we injected the c26 adenocarcinoma cell line into CD2F1 mice treated with or without minocycline, which was delivered through their drinking water. We hypothesized that minocycline treatment in tumor-burdened animals will improve cardiac function without altering MMP levels. Minocycline has already been
used in the c26 model to treat depressed mood (101); however, its ability to prevent the cardiac dysfunction is not known and could represent an attractive therapeutic in cancer treatment.

3.2 Materials and Methods

3.2.1 Animal Husbandry

Adult ~10 week old female mice weighing 20-22 grams were obtained from Charles River Laboratories (Charles River, Willmington, MA). Mice were house 1-3 per cage and were maintained on a 12 hour light/dark cycle at 25°C and were provided ad libitum access to standard rodent chow as well as water. Treated mice were administered minocycline in their drinking water which was provided ad libitum access as well. All animal care and procedures were approved by the Ohio State University Institutional Animal Care and Use Committee.

3.2.2 Model of Tumor Growth

The c26 line was maintained and injected as previously described (150). Upon arrival, mice were allowed to acclimate to their environment for one week before being randomly assigned into treatment or tumor groups. After acclimation and group assignment, 5x10⁵ c26 cells suspended in PBS were injected subcutaneously between the scapulae as described previously (150). Tumor growth was observed as early as day twelve and mice typically were cachectic and moribund by day twenty-four post injection. At the time of euthanasia, mice were anesthetized with a ketamine/xylazine
cocktail (10/1 mg/ml respectively) at a volume of 0.01 ml per gram of body weight or approximately 0.2 ml. A cardiectomy was performed, the left ventricle was dissected, snap frozen in liquid nitrogen and stored at -80°C until biochemical analyses.

3.2.3 Echocardiography

Cardiac function was determined using echocardiography with a 40 mHz VEVO 2100 Ultrasound System (Visual Sonics, Toronto, Ontario, Canada). Mice were anesthetized using isoflurane, 3% in 100% oxygen for induction and 1% in 100% oxygen for maintenance. Animals were placed on a warm table, fur around the chest was removed using a depilatory agent, and temperature was measured via a rectal probe. Pre-warmed ultrasound gel was applied to a 15 mHz transducer probe optimized for mouse echocardiography. The transducer was placed on the parasternal short axis to obtain a view of the left ventricle (LV) at the mid-papillary level for image capture and measurements. LV dimensions; LV End Diastolic Dimension, LV End Systolic Dimension (LVEDd and LVEDs), as well as posterior wall thickness during systole and diastole (PWTs, PWTd), were acquired using the leading edge method, as recommended by the American Society for Echocardiography (68). Percent fractional shortening (FS%) was calculated using the following formula; 

$$ FS\% = \left[ \frac{LVEDd - LVEDs}{LVEDd} \right] \times 100 $$

3.2.4 Cardiomyocyte Isolation

Cardiomyocytes were isolated as previously described (129). In brief, hearts were removed and digested using liberase and trypsin. Once digested, the cardiomyocytes were
plated on laminin coated glass inserts. Inserts were loaded into a perfusion chamber mounted on an Olympus IX-71 microscope. Cells were stimulated (1 Hz, 3-ms duration) with a Myopacer Field-Stimulator system and function was determined using Sarclen Sarcomere Length Acquisition Module (IonOptix, Milton, MA). Approximately 10-15 cells were measured from each mouse to provide adequate measurements for peak shortening (PS%), cellular equivalent to FS%. Sarcomere contractile velocity was also measured as ±dL/dt and time to 90% shortening and relengthening as TPS90 and TR90, respectively. For Ca\(^{2+}\) measurements, a calcium sensitive fluorimetric compound, FURA-2AM, was loaded onto the cells at a concentration of 5 µM for 20 minutes. Fluorometric measurements were acquired using a dual excitation single emission system. These transients were analyzed for transient calcium amplitude (Δ340/380), and calcium reuptake (τ).

3.2.5 RNA Isolation and qPCR

RNA was isolated from frozen tissue using the Trizol/Chloroform method. Briefly, the tissue was homogenized with a TissueLyser (Qiagen, Boston, MA) with Trizol buffer, transferred to a chloroform-containing tube and centrifuged. The supernatant was mixed with alcohol and loaded onto a Qiagen RNA processing column and the Qiagen RNA isolation protocol was followed from the Qiagen RNeasy Min-Kit (Qiagen, Bostom, MA). RNA quality and concentration were checked using a NanoDrop 2000c (ThermoScientific, Wilmington, DE), then a known amount of RNA was reverse transcribed to generate cDNA using the iScript Supermix kit (Bio-Rad, Hercules, CA).
The cDNA was diluted to an appropriate concentration and qPCR was performed as previously described (150). Briefly, the cDNA was mixed with SYBR Green Master Mix (Bio-Rad, Hercules, CA), and forward and reverse primer sequences. The resulting mix was run through a CFX96 (Bio-Rad, Hercules, CA) Three Step Amplification protocol and each cycle was repeated forty times. The Ct values of an internal control, GAPDH, were then used against the target Ct values and analyzed with the Livak $2^{\Delta\Delta Ct}$ method.

3.2.6 Western Blotting

Proteins were homogenized from snap frozen tissue using a tissue lysis buffer, as described previously (140). The protein concentration was then quantitated using the Bicinchinonic Acid Assay (BCA) (ThermoScientific, Wilmington, DE). The samples plus standards were incubated at 37°C and then read using a PowerWave plate reader (BioTek, Winooski, VT) at 562 nm. The sample concentration was extrapolated using the slope of the standard curve and was used to assure equal loading. The protein homogenate was mixed with a sample buffer and loaded onto a polyacrylamide gel (5% stacking, 12% resolving) and run at constant voltage (100v). The gel was transferred onto a PVDF membrane using a wet transfer system (Bio-Rad, Hercules, CA) that was run at room temperature for 90 minutes at 100 V. The membranes were blocked for 1 hour at room temperature with a TBS-T solution containing 5% Bovine Serum Albumin (BSA). The primary antibody was suspended at the same concentration as previously (24) used and incubated overnight at 4°C. The membrane was washed in TBST in triplicate for five minutes, then secondary antibody was applied at a dilution of 1:5000 in TBST containing
2.5% BSA for an hour at room temperature. The membrane was then washed again in TBST in triplicate for five minutes. The membrane was developed using the LiCor (Li-Cor, Lincoln, NE) imaging system.

### 3.2.7 Protein Quantitation

Immunoblots were quantitated using the Li-Cor Odyssey system (Li-Cor, Lincoln, NE). In brief, secondary antibodies with an infrared conjugate were excited using a laser system in the Li-Core Odyssey Infrared Imaging System. The emission was quantitated as an emission signal with automatic background subtraction. Additionally, a separate gel was run and stained with Coomassie Blue, where the actin region was used to ensure equal loading and normalization. The Coomassie stained gel was placed into the Odyssey system (Li-Core, Lincoln, NE) and a signal was obtained using the 700 nm channel, as previously described (45, 78). This signal was used to normalize the immunoblots; the average of the normalized signal from the control group was used to determine the fold change of the protein of interest. Three samples from each group were exposed on one immunoblot and the fold change was determined by normalization of the signal with the loading control signal and then divided by the average from the normalized control signal. Fold changes were determined this way for each immunoblot, two blots for each target protein, and the fold changes from each blot were run through statistical software together for a total of n=6 per target protein.
3.2.8 Statistical Analyses

All data are reported as the mean ±SEM with n=3-6 mice depending on the experiment. For cardiomyocyte studies 10-15 cells were used from each mouse, with 3 mice form each group for a total of 30-45 cells per group. The data were analyzed using a two-way analysis of variance (ANOVA) to determine any interactions caused by the tumor, drug, or both using Prism 6 (Graphpad Software, Inc, La Jolla, CA). A Bonferroni post hoc test was used to determine individual significant differences between groups. Differences were considered statistically significant if p<0.05.

3.3 Results

3.3.1 Minocycline treatment attenuated cardiac dysfunction

Tumor burden presents with a depressed cardiac phenotype, showing significant reduction in FS% when compared to control values (150). Tumor-bearing mice had significantly reduced posterior wall thickness during systole (PWTs), FS%, and EF when compared to untreated controls. Cardiac function was restored as determined by echocardiography in tumor mice treated with minocycline. Treatment of tumor-burdened mice showed significant improvements from untreated in FS% and EF. The PWTs of tumor-bearing mice treated with minocycline was not significantly different from control mice.
Figure 3.1: Echocardiography data taken at the parasternal short axis at the mid papillary level of control and c26 tumor-bearing mice with or without minocycline treatment. *p<0.05 was considered statistically significant from controls while #p<0.05 was considered statistically significant from c26 tumor-bearing mice.
3.3.2 Minocycline restored contractile time in isolated cardiomyocytes with no effect on calcium transient amplitude or reuptake

Cardiomyocytes from tumor-bearing mice with or without minocycline treatment showed no change in peak shortening (PS%). Additionally, there were no changes in the positive or negative velocities of contraction in tumor-burdened minocycline treated mice. The only significant change was in the time to peak at 90% of peak shortening (TPS90) and time to relaxation (TR90) of tumor-bearing minocycline treated mice. Tumor-bearing minocycline treated mice had significantly reduced calcium transients, shown as Δ340/380, the same as non-treated tumor mice. Tumor mice with or without minocycline treatment showed no difference in calcium reuptake.
Figure 3.2: Contractile properties of isolated cardiomyocytes from control and c26 tumor-bearing mice with or without minocycline treatment. *p<0.05 was considered statistically significant from controls, while #p<0.05 was considered statistically significant from c26 tumor-bearing mice. $p<0.05 shows a significant drug effect regardless of tumor-burden.
Figure 3.3: Calcium transient amplitude as well as calcium reuptake of isolated cardiomyocytes from control and c26 tumor-bearing mice with or without minocycline treatment. *p<0.05 was considered statistically significant from controls.
3.3.3 Minocycline treatment did not affect MMP or TIMP gene expression

MMP and TIMP mRNA expressions were determined in the left ventricles of tumor mice with or without minocycline treatment. In tumor-burdened minocycline-treated mice, we found that MMP-2, -3, and -14 were still significantly upregulated. MMP-9 showed a trend of increasing gene expression; however, the difference was not statistically significant when compared to untreated controls. TIMP-1 was significantly increased in tumor mice regardless of treatment, while TIMP-2 in tumor mice showed no significant increase when compared to untreated control.
Figure 3.4: RNA expression changes of MMPs and TIMPs in the left ventricle of control and c26 tumor-bearing mice, with or without minocycline treatment. RNA expression levels were determined using the Livak method with GAPDH serving as the internal control. *p<0.05 was considered statistically significant from untreated controls.
3.3.4 Minocycline treatment reduced collagen I and III gene expression in tumor-bearing mice

Collagen I and III gene expression values were significantly increased in tumor mice. When treated with minocycline, the tumor mice showed significant decreases when compared to untreated tumor mice.
Figure 3.5: RNA expression of collagen I and III in the left ventricle of control and c26 tumor-bearing mice with or without minocycline treatment. RNA expression levels were determined using the Livak method with GAPDH serving as the internal control. *p<0.05 was considered statistically significant from control, while #p<0.05 was considered statistically significant from c26 tumor-bearing mice.
3.3.5 Protein levels of MMP-2,-3, and -14 were unchanged in treated mice while MMP-9 and TIMP-2 were reduced.

Protein levels of MMP-2, -3, and -14 were significantly increased in tumor mice and minocycline treatment had no effect. However, tumor mice administered minocycline had significantly reduced levels of MMP-9, as well as TIMP-2
Figure 3.6: Protein fold changes determined by normalization with actin. *p<0.05 was considered statistically significant from controls, while #p<0.05 was considered statistically significant from c26 tumor-bearing mice.
Figure 3. 7: Representative immunoblots showing protein changes in MMPs and TIMPs from the left ventricle of control or c26 tumor-bearing mice with or without minocycline treatment.
3.4 Discussion

In our current study, we expanded upon previous research showing collagen deposition and increased MMP levels in the failing hearts of tumor-burdened mice. MMPs are increased in other models of heart failure and are correlated with morbidity in human heart failure patients (118, 125). Minocycline, a member of the tetracycline family, represents an attractive means to determine MMP contribution to the failing heart as an inhibitor of MMP activity. Treatment of tumor-bearing mice with minocycline improved posterior wall thickness (PWT) during systole, while not affecting MMP-2,-3, and -14 RNA and protein expression. Protein expression of MMP-9, and TIMP-2 were significantly reduced with the administration of minocycline. The cardiomyocytes of mice treated with minocycline, regardless of tumor burden, showed increased contractile times. Collectively, we have demonstrated that minocycline represents an attractive therapeutic for future consideration in patients with cancer cachexia.

Minocycline treated, tumor-burdened mice had significant changes in MMP-2, -3, and -14. MMP-9 was significantly reduced when compared to non-treated tumor-bearing mice. This was most likely due to the influence of MMP-3 which has been shown to regulate MMP-9 (138). With the inhibitory effect of minocycline, MMP-3 is no longer able to provoke an MMP-9 up-regulation (102). The lack of change in MMP expression was expected and experimentally beneficial as the action of minocycline could not be misinterpreted as a result of reduced MMP expression. Minocycline has been shown to have no effect on tumor growth itself, meaning the probable mechanism of minocycline is direct MMP inhibition and not due to anti-tumor effects (101). The protein expression
of TIMP-2 was decreased in minocycline treated tumor-bearing mice. The decrease in TIMPs in minocycline treated mice may be due to the lack of MMP activity, as MMPs may cleave certain transcription factors to increase TIMPs in order to rebalance the MMP to TIMP ratio. By rebalancing this MMP/TIMP ratio, MMP mediated remodeling would be attenuated or prevented altogether. In minocycline treated tumor burdened mice there were decreases in TIMP but not MMP, without MMP inhibition by minocycline this reduced TIMP expression would normally indicate increased cardiac dysfunction, which is not observed. This indicates minocycline is acting in a direct fashion on MMPs as opposed to secondary signaling, which refers to the possible anti-apoptotic and anti-inflammatory mechanisms of minocycline. A previous study demonstrated that minocycline administration to c26 tumor-bearing mice had no effect on systemic inflammation, measured as plasma concentrations of IL-6 (101). Minocycline has also been shown to have anti-apoptotic effects via attenuation of the TNF-alpha receptor (71); however, this has only been demonstrated in microglia. Since minocycline does not reduce systemic inflammation in the tumor-bearing mouse, we can infer that its cardioprotective effects are due to MMP inhibition rather than these secondary effects.

In previous studies it has been shown that there is increased collagen deposition in the hearts of c26 tumor-bearing mice (24). We examined gene expression of both collagen I and III to determine if the fibrotic response remains. In tumor-bearing mice, we found significant increases in both collagen I and III; however, in treated tumor-bearing mice we found significantly less when compared to untreated tumor-bearing mice. MMPs, through their enzymatic action, can provoke an increased collagen
deposition. Increased MMP activity begins a cycle of collagen breakdown causing increased collagen deposition as a response to fibrosis (9, 107). In minocycline treated tumor-bearing mice, this response was decreased, indicating less collagen deposition in the heart. Inhibition of MMP enzymatic activity by minocycline possibly prevented the pathological fibrotic response from occurring. Further work is required to determine if MMPs are directly provoking this response or acting as or on a signaling factor promoting collagen synthesis in the c26 model.

Our cardiomyocyte data agrees with previous findings, indicating no change in the sarcomere percent peak shortening in the c26 model of cancer cachexia (129, 150). There were significant decreases in TPS90 and TR90 in tumor-bearing mice compared to control, as the velocity was unchanged, which could indicate increased sarcomere length resulting in normalization. This effect was ameliorated when minocycline was administrated, implicating MMP activity in the sarcomere. MMP-2 has recently been shown to localize to the sarcomere and performing proteolysis on cardiac sarcomeric proteins such as, titin, myosin light chain, apha-actinin, and TnI (3). It is possible that minocycline-treated mice had restored shortening and re-lengthening times due to inhibition of MMP-2 (3). Further work will need to be performed to determine whether MMPs are altering the sarcomere. Calcium transient amplitude, or the release of calcium from the sarcoplasmic reticulum (SR), was decreased in tumor-bearing mice and unchanged when treated with minocycline. The low amount of calcium release might be indicative of posttranslational alterations to calcium handling proteins in an MMP-independent manner since this phenotype was not recovered with the administration of

82
minocycline. The rate of calcium uptake, or tau, in tumor-bearing mice was unchanged from control mice. Minocycline treatment, in control or tumor-bearing mice, had no effect on tau. This indicates that MMPs could be altering the sarcomere through proteolytic activity but the calcium dysfunction present is MMP-independent and warrants further investigation to determine how the proteins are being altered in the tumor-bearing myocardium.

Heart function is notably depressed in this cancer model with reduced FS% (129, 131, 132, 150). This reduction in the FS% is the result of thinning of the posterior wall during systole, causing chamber expansion and reducing blood ejection. The posterior wall thickness during diastole is unchanged in tumor-bearing mice, resulting in a normal diastolic chamber dimension. The increased compliance of the left ventricular wall is most likely due to the actions of MMPs. By degrading components of the ECM, the ventricle becomes increasingly compliant such that high pressure, causes a thinning of the left ventricular wall that is not present during diastole. Tumor-bearing mice treated with minocycline showed increased PWTs, becoming comparable to control levels. The improvement of PWTs improved the systolic volume, improving cardiac function.

Our results demonstrate that minocycline is capable of restoring cardiac function in a tumor-bearing mouse without altering tumor growth trajectory or inflammatory state. The most likely cause of this is the ability of minocycline to inhibit MMP activity directly and prevent enzymatic action of the MMPs. Minocycline represents an attractive therapeutic to be administered before or alongside chemotherapeutics in cancer patients to prevent cardiac complications. Administration may not only prevent cardiac
complications during treatment but also preserve quality of life post treatment by ameliorating any cardiac dysfunction. More work is still needed to further elucidate the roles that MMPs play in cardiac dysfunction in a tumor-bearing mouse. Minocycline restored contractile time in the cardiomyocyte, indicating that MMPs may function within the sarcomeric complex. Regardless, minocycline administration has demonstrated marked cardiac functional improvement and further elucidated the roles that MMPs play in the tumor-burdened murine myocardium.
Chapter 4: Increased Hypoxia Inducible Factor in Striated Muscle of Tumor-Bearing Mice

4.1 Introduction

In the United States, cancer is the second leading cause of death behind cardiovascular disease, and, in the United Kingdom, cancer is the leading cause of death (121, 137). As the quality of life index continues to increase, cancer will become a more prevalent problem in developing and third world countries (60). In the United States, approximately 40% of men and women will be diagnosed with cancer at some point in their lifetime (47). Cancer, a heavy burden on its own, can also lead to the development of cachexia (133, 134), a wasting syndrome in which muscle and adipose tissue experience marked loss and breakdown which is not nutritionally reversible. In addition to the fat and muscle wasting, there are significant appetite and metabolic complications in cachectic patients. Cachectic patients also suffer a reduced quality of life, experiencing greater fatigue, exercise intolerance, and depression (135, 136). Cachexia is inherently difficult to treat due to the lack of FDA-approved drugs and cachexia causes patients to be refractory to conventional cancer treatments. For cancer patients, there is a 30-80% chance they will develop cachexia, with certain cancers: gastric, prostate, colon, and lung cancer having high incidences of cachexia (43). Among the patients who develop cachexia, it is the primary
cause of death in 20-40% of them (134). The extensive burden and complications of cachexia warrant further investigation into the mechanisms of progression to allow development of new therapeutic strategies.

The heart is not typically considered at risk for wasting during the development of cancer-induced cachexia. However, recent research has shown that cardiac function is compromised in a tumor burdened setting. Heart samples from patients who died as a direct result of cachexia showed significant fibrotic remodeling, indicative of heart failure (127). Using an experimental model of cancer cachexia, the colon-26 (c26) adenocarcinoma cell line, it has also been shown that tumor burden can significantly impact cardiac function (150). CD2F1 mice subcutaneously injected with this cell line show reduced ejection fraction and fractional shortening with a reduction in posterior wall thickness during systole (150). Studies have implicated the ubiquitin proteasome system, autophagy, angiotensin II, and matrix metalloproteinases in this cardiac dysfunction (23, 129, 132). However, a number of possible contributors to cardiac dysfunction remain unknown. In order to uncover possible mechanisms of cardiac dysfunction, a proteomics approach was utilized. The proteomics approach, however, creates several problems that must be overcome, namely sorting and analyzing the data. A data analyzer from Qiagen was used (Ingenuity Pathway Analysis (IPA)) to sort the data and determine connections. Both metabolic and cytoskeletal differences were linked to hypoxia inducible factor 1α (HIF-1α).

HIF-1α is a transcription factor that responds to the amount of oxygen available in the environment, increasing in expression during hypoxia (142). HIF-1α is a transcription
factor for more than 60 genes (142). In normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylase-2 (PHD2); this posttranslational modification is then recognized by the ubiquitin ligase, Von Hippel-Lindau tumor suppressor (VHL). VHL ubiquinates the hydroxylated HIF-1α, marking it for destruction by the proteasome complex (53). PHD2 itself requires several cofactors in order to perform the hydroxylation reaction, namely oxygen and iron (33). In hypoxic conditions, HIF-1α expression increases as PHD2 is no longer able to mark HIF-1α for destruction. HIF-1α is known to be upregulated in human tumors, contributing to the development of large tumors which have a hypoxic core (116, 156). However, possible upregulation of HIF-1α in other tissues remains unknown.

In this project, we demonstrate, through a proteomics approach, coupled with data analysis, that HIF-1α as a possible contributor to heart and skeletal muscle pathology of tumor-bearing mice. We also demonstrate possible mechanisms contributing to HIF-1α increases and discuss possible consequences of HIF-1α up-regulation.

4.2 Materials and Methods

4.2.1 Animal Husbandry and Care

Adult female mice (~10 week old), weighing 20-22 grams, were obtained from Charles River Laboratories (Charles River, Willmington, MA). Mice were house 1-3 per cage and were maintained on a 12 hour light/dark cycle at 25°C. The mice were provided ad libitum
access to water and standard rodent chow throughout the duration of the study. All animal care and procedures were approved by the Ohio State University Institutional Animal Care and Use Committee.

4.2.2 Tumor Model
Colon-26 adenocarcinoma (c26) cells were maintained as previously described (150). Approximately one week after arrival, mice were randomly assigned to receive an injection of tumor cells or saline vehicle. Cells (5x10^5) were injected subcutaneously between the scapulae so that tumor growth would not disrupt locomotion. Tumor growth was evident as early as day seven post injection. The mice became moribund at approximately day 21. At the time of euthanasia, mice were anesthetized with ketamine/xylazine and a blood draw was performed from the right brachial artery. After blood collection, a cardiectomy was performed, the left ventricle was dissected, snap frozen in liquid nitrogen, and stored at -80°C until further biochemical analyses.

4.2.3 Proteomics and Bioinformatics
Proteomics analysis was performed by the OSU Proteomics Core, as previously described (84). Briefly, samples were marked with dye and subjected to 2-D electrophoretic separation. The gels were then scanned using a Typhoon 9400 variable mode scanner (GE Healthcare, Aurora, OH) to visualize the dye-labeled proteins. After identifying differences in control versus tumor-bearing tissues, the proteins were extracted from the gel and prepared for mass spectrometry. Capillary liquid chromatography tandem
mass spectrometry was used to identify peptide sequences. These peptide sequences were compared with the NCBI NR Database and then checked to ensure that only proteins with a Mascot score of 100 or higher were accepted. For a fully detailed description of the proteomics process, see supplementary materials and methods.

4.2.4 Proteomics Data Analysis

The Ingenuity Pathway Analysis (IPA) software (Qiagen, Boston, MA) was used to sort and analyze the proteomics data. An IPA compatible data-set from the proteomics results was uploaded into the IPA Knowledge Database and an interactive report was generated. To determine connections, the Pathway Builder software, provided as part of the IPA analysis software, was used to grow connections from the identified proteins. These networks were continually expanded and combined until common targets were identified for further analysis.

4.2.5 SDS-PAGE and Western Blotting

Proteins were isolated from sample tissue and run as described previously (25, 140). Briefly, the gels were run at a constant voltage until the tracking dye ran off the gel. Protein was then transferred onto a nitrocellulose membrane, which was blocked with BSA for an hour at room temperature. Blots were incubated with primary antibody for an hour at room temperature or 4°C. The blots were washed multiple times with TBST before the addition of secondary antibody for incubation, for an hour at room temperature. After washing with
TBST, the blots were visualized using the BCIP/NBT color reaction. Digital images of the blots were quantitated using ImageJ (NIH, Bethesda, MD) software.

For loading control, separate gels were run under the same conditions and stained using Coomassie Blue instead of being transferred. Digital images of the gels were quantitated using ImageJ (NIH, Bethesda, MD), which was used to calculate the protein fold change.

4.2.6 Iron Measurements

The left ventricle from control and tumor-bearing mice was weighed and homogenized with protein precipitation buffer (.53 N HCl and 5.3% trichloroacetic acid in HPLC water). The homogenate was boiled for 1 hour and cooled at room temperature for two minutes. The tubes were spun in an Eppendorf 5424 R centrifuge (Eppendorf, Hauppauge, NY) at 21,000 rcf for 10 minutes. The supernatant was loaded onto a spectrophotometer plate along with iron standards prepared using ferric ammonium citrate. The Ion-SL assay (Sekisui Diagnostics, Charlottetown, Prince Edward Island, Canada) was used to quantitate the amount of iron in the sample. The assay uses a colorimetric response to quantitate the amount of iron measured at 595 nm. A standard curve was used to determine the concentration of iron in the samples. This concentration was adjusted to the mass of the tissue to acquire the total iron amount in μg/g of tissue mass.
4.2.7 Blood Gas Measurements

To measure blood gas values, the iStat blood analyzer (Abbott, Princeton, NJ) was used along with the CG8+ Cartridge (Abbott, Princeton, MJ). Blood was drawn from the brachial artery and approximately 100 µl were dispensed into the cartridge sample port. Values were recorded and then analyzed for the significance of differences between groups.

Arterial oxygen content (CaO₂) is a calculated value using the equation:

\[ CaO_2 = Hb \left( \frac{\text{gm}}{dl} \right) \times 1.34 \text{ml} \frac{O_2}{gmHb} \times SaO_2 + PaO_2 \times \left( 0.003 \frac{ml-O_2}{mmHg} \right) \]

where \( Hb \) is hemoglobin, \( SaO_2 \) is the percent of hemoglobin saturated with oxygen, and \( PaO_2 \) is the oxygen partial pressure. \( Hgb, SaO_2, \) and \( PaO_2 \) were measured directly with the iSTAT.

1.34 \( \frac{ml}{gmHb} \) and 0.003 \( \frac{ml-O_2}{mmHg} \) are constants representing the amount of oxygen carried by hemoglobin and the amount of oxygen dissolved in plasma, respectively.

4.2.8 Oil Red O Staining and Quantitation

Hearts from control and tumor-bearing mice were preserved in cryo-embedding media (OCT), frozen in liquid nitrogen, and stored at -80°C until sectioning. The OCT blocks were sectioned using a cryostat and fixed onto glass slides. The sections were stained in Oil Red O stain (Abcam, Cambridge, MA) for 45 minutes. Sections were rinsed in distilled water three times. The slides were stained in Harris Hematoxylin, washed in tap water, washed in ammonia water to contrast the nuclei, followed by another wash in tap water. The slides were then mounted with aqueous mounting media.
Slide images were captured using an Olympus IX73 (Olympus, Pittsburgh, PA) at 20X magnification. The stained slides were quantitated as previously described (89). Briefly, the images were opened in ImageJ (NIH, Bethesda, MD) and converted into an 8-bit grayscale image. The threshold limit was set using a control slide and this threshold limit was applied to every slide. The measure feature on the software was used to determine the area of red staining on the slide and the area was used to perform statistical analyses as well as graphing.

4.2.9 Statistical Analyses

Values are reported as the average ± SEM for control and tumor-bearing mice. For the RNA, protein, and iron experiments, n=6 for both groups. For blood measurements, n=5 for each group. The histological analyses were n=3 for each group. To determine significant differences, a Student’s t-test was used, using Prism 6 (Graphpad Software, Inc, La Jolla, CA). Differences were considered statistically significant if p was <0.05.

4.3 Results

4.3.1 Identification of downstream and upstream effectors of HIF-1α levels

IPA was used to analyze the proteomics data. Analysis of the proteomics results indicated connections to HIF-1α. The HIF-1α suppressive protein, HSP68, was decreased in tumor-bearing mice, while several downstream HIF-1α proteins such as transferrin,
albumin, thrombin, vimentin and glutaminase were increased in tumor-bearing mice. See table 1 for the full list of proteins.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Effect on HIF-1α</th>
<th>Change</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Albumin</td>
<td>Increases HIF-1α</td>
<td>Increased</td>
<td>(95)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Increases HIF-1α</td>
<td>Increased</td>
<td>(42)</td>
</tr>
<tr>
<td>Transglutaminase</td>
<td>Increases HIF-1α</td>
<td>Increased</td>
<td>(66)</td>
</tr>
<tr>
<td>Transferrin</td>
<td>HIF-1α increases expression</td>
<td>Increased</td>
<td>(151)</td>
</tr>
</tbody>
</table>

Table 4. 1: Proteins detected as interacting with HIF-1α through the Ingenuity Pathway software.

4.3.2 Increase in HIF-1α and cKit protein levels in the myocardium

HIF-1α was significantly increased in tumor-bearing mice, as determined by western immunoblots. The regulatory elements PHD2 and VHL were unchanged between control and tumor-bearing mice. Additionally cKit was slightly, but significantly, increased in the myocardium.
Figure 4.1: Protein fold changes in the left ventricle determined through normalization with actin loading control. *p<0.05 was considered statistically significant from controls.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHD2</td>
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<td><img src="image.png" alt="Immunoblot Image" /></td>
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<tr>
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<td><img src="image.png" alt="Immunoblot Image" /></td>
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<td><img src="image.png" alt="Immunoblot Image" /></td>
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<tr>
<td>cKit</td>
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<tr>
<td>Actin</td>
<td><img src="image.png" alt="Immunoblot Image" /></td>
<td><img src="image.png" alt="Immunoblot Image" /></td>
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</tbody>
</table>

Figure 4.2: Representative immunoblot of protein expression changes in the left ventricle of control and tumor bearing mice.
4.3.3 Decreased iron concentration in the myocardium

Iron concentration was significantly decreased in the myocardium of tumor-bearing mice when compared to controls.

Figure 4. 3: Quantitation of iron concentration in the myocardium determined by acid release assay in control and tumor mice. *p<0.05 was considered statistically significant.
4.3.4 Decreased oxygen levels in arterial blood

The partial pressure of oxygen and the concentration of oxygen were significantly decreased in tumor-bearing mice, with no changes observed in hemoglobin or hematocrit levels.
Figure 4.4: Blood gas measurements from arterial blood of control and tumor mice measured through the iStat Bio-Chip reader. *p<0.05 was considered statistically significant.
4.3.5 Increased total carbon dioxide and calcium in arterial blood

The total carbon dioxide content was significantly increased in the tumor-bearing mice when compared to control mice. Several ions were also measured and only calcium was found to be significantly increased in the tumor-bearing mice.
Figure 4.5: Measurements of total carbon dioxide and ionic concentrations in the arterial blood of tumor and control mice determined through the iSTAT Bio-Chip reader. *p<0.05 was considered statistically significant.
4.3.6 Increased lipid deposition in the myocardium

Staining with Oil Red O revealed significant lipid deposition in the myocardium of the tumor-bearing mice when compared to control mice.
Figure 4.6: Quantitation of Oil Red O stained slides from the left ventricle of control and tumor bearing mice. *p<0.05 was considered statistically significant.
4.3.7 Increase in HIF-1α protein levels in select skeletal muscle

HIF-1α was found to be significantly increased in the soleus, EDL, and diaphragm of tumor-bearing mice.
Figure 4. 7: HIF-1α protein fold changes in skeletal muscle of control and tumor mice. *p<0.05 was considered statistically significant.
Figure 4. 8: Representative immunoblots of HIF-1α expression changes in skeletal muscle with loading controls.
4.4 Discussion

Our findings demonstrate increased HIF-1α in tumor-burdened mice in the heart, EDL, soleus, and diaphragm. While proteomics was originally only performed on the heart, leading to the focus on HIF-1α, it became evident that other tissues might be affected as well. Finding increased amounts of HIF-1α, without compensatory increase in PHD2 or VHL, necessitated looking for a plausible mechanism to explain the increased HIF-1α. Since PHD2 requires both oxygen and iron as a cofactor to hydroxylate HIF-1α, so that it can be recognized by VHL for ubiquination, we determined the amount of oxygen through blood gas measurements as well as the amount of iron present in the myocardium. There were significantly lower oxygen pressure and concentration in the arterial blood of tumor-bearing mice, indicating a hypoxic environment. We also examined the EDL, soleus, and diaphragm and all were found to have significantly increased expression of HIF-1α. The effect of hypoxia and subsequent increase in HIF-1α in the heart has both beneficial and detrimental effects. Transient hypoxia and induction of HIF-1α has been shown to be cardioprotective in high altitude training and in myocardial infarction (64, 104, 149). A plausible mechanism for this is HIF-1α regulates oxygen consumption of mitochondria, providing protection during hypoxic events and decreased oxygen availability (117). Sustained hypoxia and a subsequent increase in HIF-1α are associated with detrimental effects on the heart (41). The lack of available oxygen impacts beta-oxidation of fatty acids, preventing lipids from being utilized as an energy source. This leads to lipid accumulation in the myocardium (18, 100, 105). Our results confirm that there is lipid accumulation in the hearts of tumor-bearing mice, which could contribute to cardiac dysfunction through
lipid toxicity. Lipid accumulation typically results in an increase in PPARα; however, hypoxia has been shown to decrease PPARα expression with a consequent shift from lipid to glucose utilization (97). PPARα has been shown to be decreased in the c26 model of cancer cachexia (131). Our results indicate that hypoxia may be responsible for this decrease. HIF-1α is associated with numerous stem cell expression factors possibly as a protective mechanism. In the heart, c-Kit has been implicated as a progenitor marker leading to cardiomyocyte differentiation (80, 112). Previous studies have shown that hypoxia and the action of HIF-1α can induce c-Kit expression, possibly as a protective mechanism to replenish apoptotic cells as a result of hypoxia (77). Our findings show an increased amount of c-Kit in the myocardium of tumor mice, indicating that HIF-1α may be involved in progenitor cell activation.

Due to the decreased oxygen concentrations in the arterial blood of tumor-bearing mice, we determined if HIF-1α was also increased in skeletal muscle. Our findings show that HIF-1α is significantly increased in the EDL, soleus, and diaphragm. This indicates that there might be a hypoxic response regardless of fast (EDL) or slow (soleus) muscle type. The role of HIF-1α in skeletal muscle is not as well understood when compared to its role in the myocardium. Most of the available knowledge is based on endurance and exercise training studies, where transient increases in HIF-1α promote increased oxidative capacity, angiogenesis, and muscle repair (5, 51). However, HIF-1α expression quickly returns to normal after the induction of repair and regeneration. Chronic cardiac hypoxia is associated with decreased mitochondrial capacity and content (50). In the c26 model, skeletal muscle have distorted mitochondria as determined by electron microscopy,
although no studies have been reported on the oxidative capacity of these mitochondria (119). The diaphragm in mice is primarily composed of fast fibers and also shows increased expression of HIF-1α, similar to the EDL and soleus. This represents a paradox, where the diaphragm would be needed to increase respiration to restore oxygen levels to normal. It was thought that the diaphragm might have protective mechanisms to maintain function during hypoxia. However, a study involving exposure of mice to an hypoxic environment showed that the diaphragm has reduced mitochondrial capacity and integrity (40). The most common cause of death of cachectic patients is respiratory failure. While muscle weakness is most likely the primary cause, HIF-1α may contribute through the suppression and loss of mitochondria (134).

A limitation of our study is that we currently have no mechanism to explain the decrease in oxygen pressure and concentration associated with tumor burden. Hypoxia and HIF-1α have been extensively studied in relation to the tumor and its hypoxic core, but there is little information on hypoxia experienced by patients affected by cancer. One possible explanation is that there was no change in the pressure of carbon dioxide (pCO₂) in the tumor-bearing mice compared to control mice. Despite the reliance on oxygen, the body regulates its respiration rate through the sensing of carbon dioxide (98). Without a change in the pCO₂, there may be no change in respiration, advancing the hypoxic phenotype. We also found significantly increased calcium, bicarbonate (HCO₃⁻), and base excess (BE) in the arterial blood of tumor-bearing mice. The increase in calcium has been associated with increased hypoxia as a result of calcium moving from the intracellular to the extracellular space (8). The increase in HCO₃⁻ and BE could indicate metabolic
alkalosis which is normally compensated by hypoventilation. The respiratory ability in cancer patients may be compromised. Renal compensation, while less efficient, is another method to restore normal bicarbonate levels. With increased calcium, as well as HCO3 and BE, it seems evident that the kidneys are no longer adequately filtering the blood.

Our findings demonstrate a novel pathway in the setting of cancer-induced cachexia and reveal the possibility of new mechanisms, as well as therapeutic targets. We show that HIF-1α is increased in several striated muscles and is likely the result of a systemic hypoxic phenotype in the c26 model. These findings also implicate other, possibly physiologically relevant systems, such as the respiratory and renal systems. The heart was thought to be spared the wasting experienced during cancer cachexia, a thought that has recently been refuted by multiple models as well as human studies (127, 131, 132, 150). Cancer cachexia could be causing unknown effects in tissue types, which if explored could provide new mechanisms and therapeutic treatments for a wide range of cachexia patients. Our findings show that there could be systemic effects due to hypoxia and produce new mechanistic/therapeutic insight.
Chapter 5: Alterations to Calcium Handling Proteins in the Hearts of Tumor-Bearing Mice

5.1 Introduction

Cancer is an ever increasing health concern, especially among developed countries such as the United States and the United Kingdom. In the United States, approximately 1 in 3 people will develop cancer, with the majority of diagnoses occurring over the age of 55 (121, 137). Cancer can be complicated by the development of cachexia, an aggressive wasting syndrome. Cancer-induced cachexia is the aggressive breakdown of both adipose tissue and skeletal muscle, resulting in significant loss of body mass. Approximately 1 in 2 cancer patients will develop cachexia, with the figure rising to over 80% in patients with advanced stage cancer. Patients who develop cachexia become resistant to conventional cancer treatments. Increasing the likelihood of death as a result of cancer. These patients also face increased mortality risk from cachexia. Cachexia will be the direct cause of death for 1 in 4 patients with this syndrome. While the heart was not originally thought to be affected by this wasting syndrome, recent studies in experimental animals and humans have revealed this is not the case (133, 134, 150).

The heart during cancer induced cachexia has been shown to present with symptoms similar to heart failure. Recent experimental findings in animal models of
cancer cachexia have shown compromised myocardial function (131, 132, 150). There is emerging evidence for the involvement of the ubiquitin ligase system in the hearts of mice with cancer induced cachexia, possibly caused by the effects of Angiotensin-II (Ang-II). Ang-II is an activator of the ubiquitin ligase pathway, a pathway that is responsible for protein degradation. Treatment with Ang-II receptor blocker (ARB) was shown to prevent cardiac dysfunction (129). Ang-II also mediates fibrotic remodeling through the activation of matrix metalloproteinases (MMPs), which remodel the extracellular matrix (ECM). The c26 model of cachexia was found to have significantly increased amounts of MMP in the myocardium along with fibrotic remodeling (24). MMP involvement was corroborated by the administration of minocycline, an MMP inhibitor, which prevented cardiac dysfunction. In the c26 model, cardiac dysfunction presents with altered contraction times at the cardiomyocyte level, along with significantly less calcium release from the sarcolemmal endoplasmic reticulum (SR) (150).

Alterations in the contractile kinetics and reduced calcium release from the SR indicate alterations to the proteins involved in the calcium release and management. The effects of tumor burden on the calcium handling effects of the heart are currently not known. Calcium release and contraction can be modified by the amount of posttranslational modification of their handling proteins, increasing their responsiveness. Normally this allows the heart to make rapid adjustments to contractile ability as determined by need, such as strenuous exercise. In heart failure, however, these modifications can negatively impact function by reducing the ability of the heart to
change in response to increased demand. By examining the calcium handling proteins in an experimental model of cancer cachexia we hope to uncover new mechanisms that have not been investigated.

5.2 Materials and Methods

5.2.1 Animal Husbandry

12 week old female CD2F1 mice were obtained from Charles Rivers (Willmington, MA) and were housed in animal facilities at The Ohio State University under a 12 hour light and dark cycle, maintained at 37 °C. Mice were provided ad libitum access to water and standard rodent chow. All protocols pertaining to the mice were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University

5.2.2 Murine Tumor Model

Cells were maintained as previously described (150). Briefly, the Colon-26 Adenocarcinoma (c26) cells were maintained in RPMI 1640 media supplemented with glutamine and penstrep. Before injection the cells were trypsinized, pelletized through centrifugation, and resuspended in PBS. Cells were counted and a dilution was made for animal injection so that the number of cells injected subcutaneously was 5x10^6. Before injection, the animals were anesthetized with 5% isoflurane and were maintained at 1% isoflurane during injection. Cells were injected subcutaneously in between the scapulæ of the mouse. The mice were returned to their home cage and observed to ensure their
complete recovery from anesthesia. Tumor growth was evident approximately 7-12 days post injection and the mice were moribund by day 21 post injection. Mice were euthanized using a ketamine/xylazine cocktail and tissue was harvested and snap frozen in liquid nitrogen for further processing.

5.2.3. RNA Isolation and qPCR

RNA was isolated from left ventricular tissue as previously described (129). Briefly, tissue was homogenized in Trizol Reagent (Sigma Aldrich, St. Louis, MO) using a TissueLyzer (Qiagen, Valencia, CA) with 5 mm stainless steel beads. After homogenization RNA was precipitated using chloroform to isolate RNA in the aqueous phase. The RNA was further isolated and purified using the RNEasy Extraction Kit (Qiagen, Valencia, CA). RNA was measured using a NanoDrop (ThermoScientific, Waltham, MA) to standardize the amount of RNA for cDNA synthesis. CDNA was synthesized using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). After cDNA synthesis, the cDNA was mixed with the appropriate template as well as SYBR Green Master Mix (Bio-Rad, Hercules, CA) and placed into the CFX96 (Bio-Rad, Hercules, CA) using a three step amplification protocol, as described previously (130). The results were analyzed in reference to the standard, GAPDH, using the Livak 2^(-ΔΔCt) method to determine expression changes.
5.2.4. Western Blotting and Detection

Proteins, except for RyR or PLN, were run using a pre-cast 4%-15% TGX gel (Bio-Rad, Hercules, CA) at 200 V for 30 minutes using the Bio-Rad Basic Power Pack (Bio-Rad, Hercules, CA). After running, the gels were incubated in Tris-Glycine transfer buffer for 30 minutes before being transferred onto methanol activated PVDF membranes, using the semi-dry system (Bio-Rad, Hercules, CA) at 10 V for 30 minutes.

RyR was run on an 8-12% Tris-Acetate Gradient Gel (Novus Biologicals, Littleton, CO) at 200 V for 4 hours. The proteins were transferred onto methanol activated PVDF membranes using a wet transfer system (Bio-Rad, Hercules, CA) at 10 mA overnight.

PLN was run on a 10-20% Tris-Tricine Gel (Bio-Rad, Hercules, CA) at 200 V for 1 hour. The gels were transferred onto an activated PVDF membrane using a semi-dry transfer system (Bio-Rad, Hercules, CA) at 10 V for 30 minutes.

Regardless of transfer method or gel type, the membranes were blocked using TBS Odyssey Blocking Buffer (Li-Cor, Lincoln, NE) for 1 hour at room temperature. The blots were incubated in primary antibody suspended in 2.5% BSA at 4°C overnight, then washed 3 times for 5 minutes using TBST. Near-infrared secondary antibodies (Li-Cor, Lincoln, NE) were suspended in 2.5% BSA and the blot was incubated for 1 hour at room temperature. The blots were then washed with TBST 3 times before protein detection using the Li-Cor Odyssey XL Laser Detection System (Li-Cor, Lincoln, NE). Emission values were recorded for the detected bands and used to quantitate the relative amount of expression. A separate 4%-15% TGX (Bio-Rad, Hercules, CA) precast gel
was run and stained with Coomassie R250 for 2 hours at room temperature. The gel was then placed in destain buffer until the actin band was clearly visible with minimal background. This gel was placed into the Li-Cor Odyssey XL Laser Detection System (Li-Cor, Lincoln, NE) and detected using the 800 nm laser. The emission values were then used to calculate the fold change of the protein of interest.

5.2.3. Assessment of Myosin Heavy Chain Composition

Left ventricular homogenates were prepared from control and tumor-bearing mice, as described previously (108). SDS-PAGE was used to separate the myosin heavy chain isoforms in the left ventricular samples. Separating gels consisted of 7% acrylamide, with a 50:1 acrylamide:bis-acrylamide crosslinking ratio, and 5% (v/v) glycerol, as described previously. Gels were run for 21 hours at a constant voltage of 230 in the Hoefer SE600 Large Vertical Slab Gel Electrophoresis unit that was cooled with circulating water to 8°C. After running, the gels were stained using the sensitive silver stain method as described previously. Once staining was finished the reaction was neutralized and an image was obtained for densitometry scanning with ImageJ to compare the amount of each isoform.
5.2.5 Statistical Analyses

Results from qPCR and protein fold changes were put into the statistical software Prism (GraphPad, La Jolla, CA) for statistical analysis, as well as graphing. Results from each study were analyzed using a Student’s T-Test. Results were considered significant if \( p<0.05 \) and are indicated graphically by an asterisk.

5.3 Results

5.3.1 Reduced gene expression levels of PLN

qPCR revealed significant decrease of PLN gene expression in tumor burdened mice, no other calcium handling proteins were determined to be significantly changed at the gene expression level.
Figure 5.1: RNA expressions of RyR, PLN, Cav1.2, and SERCA in the left ventricle of control and tumor mice. RNA expression levels were determined using the Livak method with GAPDH serving as the internal control. *p<0.05

5.3.2 No change in PLN or phosphorylated-PLN (pPLN) or PLN/SERCA ratio

PLN and pPLN were not found to be significantly changed between control and tumor bearing mice. Additionally there was no change in the protein expression of SERCA and the ratio of PLN to SERCA was unchanged between control and tumor burdened mice.
Figure 5. 2: Protein expression of PLN, p-PLN, SERCA and the ratio between PLN/SERCA in the left ventricle of control and tumor mice. *p<0.05 was considered statistically significant.

5.3.2 Increased RyRanondie Receptor phosphorylation at Serine-2808

pRyR(S2808) was found to be significantly increased in tumor bearing mice when compared to control mice. The ratio of RyR to pRyR was found to be significantly increased in tumor bearing mice as well. There was a significant difference in FKB12 (Calstabin) expression between control and tumor mice.
Figure 5.3: Protein expression levels of RyR, pRyR, and FKB12 in the left ventricle of control and tumor mice as well as the ratio of pRyR/RyR. *p<0.05 was considered statistically significant.

5.3.3 Increased L-Type Calcium Channel protein expression

L-Type Calcium Channel protein expression was found to be significantly increased in tumor bearing mice when compared to control mice.
Figure 5.4: Protein expression change of the L-Type calcium channel in the left ventricle of control and tumor mice. *p<0.05 was considered statistically significant.
Figure 5.5: Representative immunoblots of calcium handling proteins as well as loading control in the left ventricle of control and tumor mice.
5.3.4 No change in the amount of TnI or pTnI, or changes in the ratio of $\alpha$ to $\beta$ myosin heavy chain

We found no changes in the absolute expression of TnI and pTnI as well as their ratio. Additionally there were no significant changes in the ratio of $\alpha$ to $\beta$ myosin of tumor mice when compared to control.

Figure 5. 6: Protein expression of TnI, p-TnI, their ratio and MHC isoform in the left ventricle of control and tumor mice.
Figure 5.7: Representative immunoblots of TnI, p-TnI, and MHC isoform in the left ventricle of control and tumor mice.
5.4 Discussion

Our findings indicated changes of the proteins involved in calcium induced calcium release. We found significantly increased levels of L-Type calcium channel and significantly increased phosphorylation of RyR. We did not find any changes in PLN or SERCA, which reinforce previous studies showing no change in the rate of calcium uptake in tumor bearing mice. The contraction of the sarcomere is dependent on calcium, as well, and previous results have indicated changes in the contractile kinetics of isolated cardiomyocytes in tumor-bearing mice. To this end, we examined the levels of TnI, pTnI and the isoform changes of the myosin heavy chain. There were no significant differences in TnI, pTnI, or myosin isoform composition. However, previous studies have shown increased β-MHC, as well as decreased TnI protein levels in tumor-bearing mice (129, 132). It is possible there exist differences in the heart between cachexia and what is termed severe cachexia, in which there is a higher amount of weight loss compared to cachexia. This difference between cachexia and severe cachexia has been described in the functional abilities of skeletal muscle but there has been no study to compare the differences of cachexia and severe cachexia in the heart (93). Regardless, our findings possibly indicate the presence of a leaky calcium channel, increased amounts of phosphorylated RyR could cause calcium leak from the SR, increasing the amount of cytosolic calcium.

Leaky calcium channels are a component of cardiac dysfunction in heart failure. The increase in cytosolic calcium increases the risk of sudden cardiac death from stress such as exercise or emotional distress (72). Increased levels of cytosolic calcium can also
increase calcium induced proteolysis which has been shown to occur in the AH-130 rat model of cancer cachexia. There have been no studies in the c26 model to implicate this pathway, however. The hyperphosphorylation of RyR has been implicated as an important mediator in heart failure in other models and was shown to be prevented in a RyR mutant lacking the normal phosphorylation site (52, 109). The implication of leaky calcium channels affecting heart function has been confirmed in human studies, as well. Isolated hearts from humans in heart failure were treated with beta-blockers showed improved calcium channel function when compared to failing hearts without beta-blockers. A limitation of our study is the lack of immunoprecipitation results in relation to RyR. FKB12, calstabin, was found to be significantly decreased; however, this was determined with total protein amounts. Calstabin binds and regulates the amount of calcium release from RyR, and reductions in the amount of calstabin can also contribute to leaky calcium channels. While the total protein amount may not be altered, the amount of FKB12 binding to RyR might be changed, contributing to the leaky channel hypothesis.

Contractile times of the cardiomyocyte are changed in the tumor-bearing mouse (162). Our findings here indicate that there could be alterations to the calcium handling proteins. We also show that there is no change in the TnI, pTnI, or MHC isoform composition, indicating the contractile time changes cannot be solely due to the interactions between the tropomyosin, thin, and thick filaments. It should be stated, however, that previous results show increased amounts of β-MHC isoform composition that correspond with an increased contractile time due to the slower ATP activity of β-
MHC compared to α-MHC. Again this could be due to differences between cachexia and severe cachexia and warrant further investigation. The leaky channel hypothesis is still preliminary and requires further validation. One possible method would be the use of β-blockers which have not been utilized before in the c26 model of cachexia. Our findings present new pathologies associated with the heart during tumor burden and will hopefully lead to new therapeutic strategies that can improve patient outcome and quality of life.
Chapter 6: General Discussion and Future Directions

6.1 Matrix Metalloproteinases and Cardiac Dysfunction

Finding increased matrix metalloproteinases in the cardiac and skeletal muscle of tumor mice is a novel finding and has not been shown in cancer-induced cachexia before. Increased expression of MMPs can degrade the extracellular matrix, changing the cell-to-cell communication, as well as mechanical integrity. In the heart, which is a high pressure system, maintaining this mechanical integrity is necessary for function.

Echocardiographic results from c26 tumor mice show decreased posterior wall thickness during systole only, with no compromise in diastolic function. MMP activity can weaken the extracellular matrix so that the wall under high pressure, during systole, is compressed and function is reduced. Despite finding increased MMP activity, these initial results did not deal with MMP function directly. To examine their contribution we used an MMP inhibitor, minocycline. Minocycline is a broad spectrum inhibitor of MMPs and was shown to improve cardiac function in tumor mice, restoring posterior wall thickness during systole. One issue with minocycline results is that minocycline is a broad spectrum MMP inhibitor. MMPs are capable of activating numerous other MMPs as well as MMPs activating latent MMPs of the same type. Future studies will need to utilize MMP KO mice in order to determine the specific contribution of MMPs to cardiac dysfunction. MMP-2 and MMP-9 single and double KO mice already exist; however,
there are complications with MMP KOs. MMP KO studies often find that there will be a compensatory increase in another similar MMP to compensate for the loss of function. It may be more efficient then to study tumor induced cardiac dysfunction in a tissue inhibitor of metalloproteinase (TIMP) overexpression model. By utilizing the natural inhibitory properties of TIMPs on MMPs, it would be possible to circumvent MMP KO complications and still determine their contribution to cardiac dysfunction.

6.2 Hypoxia Inducible Factor in Tumor Mice

Increased HIF-1α expression in the heart and skeletal muscle of tumor-bearing mice is a novel finding. HIF-1α has been extensively studied as part of tumor biology as increased expression is necessary to sustain the hypoxic tumor core. The expression of HIF-1α has not been examined in any other tissue in the setting of cancer as far as we are aware at the time of writing. HIF-1α is a transcription factor involved in a large number of genes related to cell survival, proliferation, and metabolism. In the tumor-bearing mouse it seemed evident that there was a decreased systemic oxygen concentration, which would promote the stabilization and overexpression of HIF-1α. Our findings show increased HIF-1α expression, a mechanisms to explain increased concentration, and possible downstream effects of HIF-1α. A limitation of our current study is that we have not investigated the therapeutic implications of HIF-1α by preventing its function. Future studies will need to utilize treatments to prevent the activity and expression of HIF-1α to determine its contribution to the pathology of tumor mice. HIF-1α KO models, while they exist, would likely not be suitable for cancer study. The only currently commercially
available HIF-1α inducible KO is a floxed gene that requires Tamoxifen to remove the floxed gene. Tamoxifen is an anti-cancer agent and could complicate the findings in a cancer model. A more systemic approach might be useful through hyperbaric treatment. Hyperbaric oxygen treatment could increase the oxygen concentration in the blood, allowing HIF-1α to be hydroxylated and marked for destruction. This would be the best approach in the c26 model, as we have shown that the regulatory enzymes, PHD2 and VHL, are intact. The necessary cofactors, oxygen and iron, are decreased, preventing PHD2 from effectively marking HIF-1α.

6.3 Calcium Handling and Cardiomyocyte Studies

The calcium protein results from our study show what would be considered a leaky calcium channel phenotype. Investigation into the calcium handling proteins was initiated through isolated cardiomyocyte findings. Isolated cardiomyocytes show faster contractile times, decreased calcium transient amplitude with normal calcium reuptake. The shorter contractile time could be the result of alterations in the calcium handling proteins. The changes in the Ryanodine receptor may effect the troponin complex, changing the contractile kinetics. In order to rule out increased troponin sensitivity we examined the total amount of TnI and phosphorylated TnI. No changes were found in either the total level of TnI or phosphorylated TnI, indicating that the faster contractile effect is not due to changes in TnI. These results are preliminary and more research will need to be done before the contractile changes and calcium amplitude can be understood. We did find increased phosphorylated RyR as well as reduced Calstabin expression in the
heart, contributing to the leaky calcium channel hypothesis. Pharmacological studies might be beneficial to uncover the contribution of RyR changes through the use of β-Blockers. B-Blockers are a class of drugs that blunt the ‘fight or flight’ response and have been effective in treating heart failure by reducing heart rate and phosphorylated RyR. With reduced heart rate and decrease phosphorylated RyR there, may be reduced calcium concentration outside the SR which would restore the contraction times in isolated cardiomyocytes pointing to a possible mechanism.

6.4 Future Experiments

There is still a significant amount of work to be done in regards to cardiac dysfunction in the setting of cancer. The research presented is very novel but also preliminary. We have examined two possible mechanisms, MMPs and HIF-1α, which have not been investigated in the setting of cancer-induced cachexia before and found significant increases. As have been mentioned in other reviews and papers, the mechanisms of cancer-induced cachexia are multi-layered and several pathways are simultaneously activated. There will likely not be one single exploitable mechanism in the treatment of cardiac dysfunction caused by cancer and there is still a great deal of basic research needed.

While echocardiography has been invaluable in determining cardiac dysfunction in the tumor mouse, cardiac dysfunction does not appear until approximately 14 days post injection in a 21 day model. Speckle tracking, another echocardiographic method, examines the twisting of the heart and can provide information about wall strain. This
method can often detect wall strain before conformational changes that would be detectable by conventional echocardiography. Using this method would enable us to determine if there is evidence of dysfunction preceding overt cardiac remodeling. Not only would this be beneficial in the c26 tumor model to determine how early changes to wall stress develop compared to cardiac dysfunction, but it would provide a reference for physicians to use the speckle tracking method to track at-risk patients and catch cardiac changes before heart failure occurs.

Isolated cardiomyocyte studies have proved invaluable in understanding the mechanisms associated with cancer induced cardiac dysfunction. The inherent problem with isolated cardiomyocytes is the lack of mechanical stimulation of the individual cardiomyocytes. Cardiomyocytes normally exist tightly connected and experience mechanical stress through the mechanoreceptors which in turn modulate their contractile properties. We can approximate the preload and mechanical stress experienced by cardiomyocytes through the use of a recent IonOptix system utilizing glass rods attached to cardiomyocytes which can then be stretched to a certain degree. Through stretching the cardiomyocytes, we effectively imitate a preload which may then show differences in the contractile ability of the cardiomyocyte. This would help examine contractile properties under a better approximation of the native state, possibly uncovering new mechanisms to be exploited.
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Appendix A: Proteomics Methods and Results

Materials and Methods:

Sample Prep:
Samples were received frozen and stored at -80°C until further processing. Proteins were extracted from cardiac tissue with 12.0 μL RIPA buffer/mg tissue using a micropustle. Probe sonication was performed for 2 x 10 sec. and the supernatant was collected. An additional extraction was performed on the remaining pellet with 2 μL lysis buffer/mg tissue (30M Tris pH 8.5, 7M Urea, 2M Thiourea, 4% CHAPS). After clarification, the extracted proteins were combined and protein concentration was determined by Bradford assay using BSA as a standard. 2D cleanup was performed on 100 μg of sample according to the manufacturer’s instructions (GE, 80-6484-51). Finally, samples were suspended in 100 μL of lysis buffer and quantitated by Bradford assay using BSA as a standard.

Sample Labeling:
50 μg of each sample was labeled with the appropriate CyDye (either Cy3 or Cy5, see Table 1). Labeling reactions were performed on ice, light-protected to avoid dye deterioration and were carried out with a ratio of 400 pmol Dye/50 μg sample. 27 μg of
each sample (325 µg total) was pooled to create an internal standard (IS) and this pooled sample was labeled with Cy2 (2.6 nmol). After 30 minutes, lysine was added to quench the reaction. Additionally, 50 µg of each sample (500 µg total) was left unlabeled and combined to run preparative gels (2 gels, 250 ug/ea) for spot-picking proteins of interest.

2D Electrophoresis: 1st Dimension

Labeled samples were combined as shown in Table 1, vortexed, and diluted with rehydration buffer (7M Urea, 2M thiourea, 2% CHAPS, 1% pH 3-10 IPG buffer (GE Healthcare), 50mM DTT, 1% saturated bromophenol blue solution) to a final volume of 450 µL. Samples were centrifuged for 20 minutes at 4 degrees Celsius/16,000 RCF, and each sample was then used to rehydrate a 24cm immobiline pH 4-7 IEF strip (GE Healthcare) under mineral oil overnight. The IEF strips were then focused on an IPGphor II (GE Healthcare) in a ceramic manifold at 20 degrees Celsius. Focus was set at 75 µA per strip maximum, focused at 500V for 1 hour, a gradient to 1000V over 1 hour, a gradient to 10,000V over 3 hours, and a hold at 10,000V for 3.25 hours. IEF strips were placed in plastic, wrapped in aluminum foil and stored at -80 degrees Celsius until further processing.

2D Electrophoresis: 2nd Dimension

Strips were equilibrated at room temperature in 5ml of equilibration buffer A (50mM Tris pH 8.8, 6M urea, 30% glycerol, 2% SDS, 0.5% DTT w/v) for 15 minutes, followed by 5ml of equilibration buffer B (50mM Tris pH 8.8, 6M urea, 30% glycerol, 2% SDS, 4.5%
iodoacetamide w/v). The reduced and alkylated strips were rinsed briefly in 1x SDS-PAGE running buffer (50mM Tris, 384 mM glycine, 0.2% SDS) and placed in 20x24cm 12% SDS-PAGE gels. Strips were sealed in place using 0.5% agarose in 2x SDS-PAGE running buffer containing 1% saturated Bromophenol blue solution. Gels were run in a Dalt 12 electrophoresis system (GE Healthcare) at 2 watts per gel for 45 minutes, followed by 15 watts per gel until the dye front reached the bottom (~4 hours). Gels (still in glass plates) were rinsed with water and immediately scanned in a Typhoon 9400 variable mode scanner (GE Healthcare) using the appropriate settings for CyDye fluorophors, scanning for cy2, then cy3, and then cy5 labels. Gels were prescanned at 1000 micron resolution to optimize PMT for each dye and then scanned at 100 micron resolution for final images. Gel images were cropped for analysis and saved. Preparative gels (for spot picking and identification) were fixed and stained with Lava purple general protein stain (Gel Company) according to standard protocols.

In Gel Digestion
The Ettan Spot Handling Workstation was used to core protein spots of interest using a pick list and placed in a 96 well plate (User Manual from Amersham Biosciences). In gel digestion was also performed by the workstation. Gel pieces were washed in 100 µL of 50% methanol/5% acetic acid for 15 min. The wash step was repeated a total of 2 times. The gels spots were washed with acetonitrile for 5 minutes, then dried and resuspended with 50mM ammonium bicarbonate; this was repeated three times. Dried gels pieces
were digested with sequencing grade trypsin from Promega (Madison WI). The protease was driven into the gel pieces by rehydrating them in 50 µL of sequencing grade-modified trypsin prepared at 5 µg/mL in 50 mM ammonium bicarbonate for 3 hours at 37ºC. The peptides were extracted from the polyacrylamide with 50 µl 50% acetonitrile and 5% formic acid three times. The extracted pool was dried for 30 minutes in a vacufuge and removed immediately to prevent complete drying.

Mass Spectrometry

*LTQ*

Capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was an UltiMate™ Plus system from LC-Packings A Dionex Co (Sunnyvale, CA) with a Famos autosampler and Switchos column switcher. The solvent A was water containing 50mM acetic acid and the solvent B was acetonitrile. 5 microliters of each sample was first injected on to the trapping column (LC-Packings A Dionex Co, Sunnyvale, CA), and washed with 50 mM acetic acid. The injector port was switched to inject and the peptides were eluted off of the trap onto the column. A 5 cm 75 μm ID ProteoPep II C18 column (New Objective, Inc. Woburn, MA) packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2-80%B over 50 minutes, with a flow rate of 300 nl/min. The total run time was 60 minutes. The MS/MS was acquired according to
standard conditions established in the lab. Briefly, a nanospray source operated with a spray voltage of 3 KV and a capillary temperature of 200°C is used. The scan sequence of the mass spectrometer was based on the TopTen™ method; the analysis was programmed for a full scan recorded between 350 – 2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the ten most abundant peaks in the spectrum. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 30 s, exclusion duration of 350 s and a low mass width of 0.5 and high mass width of 1.50 Da.

**Bioinformatics**

Sequence information from the MS/MS data was processed by converting the raw dta files into mascot generic files (.mgf) using MS Convert (ProteoWizard). The resulting mgf files were searched against all *Mus musculus* proteins in the Swiss Prot database (16,665 sequences) using Mascot Daemon by Matrix Science version 2.2.1 (Boston, MA). The mass accuracy of the precursor ions were set to 1.8 Da given that the data was acquired on an ion trap mass analyzer and the fragment mass accuracy was set to 0.8 Da. Considered modifications were methionine oxidation, deamidation (variable) and carbamidomethyl cysteine (fixed). Two missed cleavages for the enzyme were permitted. Peptides with a score less than 20 were filtered and proteins identified required bold red peptides. Protein identifications were checked manually and proteins with a Mascot score of 100 or higher with a minimum of two unique peptides from one protein having a -b or -y ion sequence tag of five residues or better were accepted.
Results:

Gel images were loaded into SameSpots software (TotalLabs) and analyzed individually (Figure 1). All saturated spots, dust spots, and noise were removed before gel normalization with manual verification that quality protein spots were not removed. SameSpots determined which gel was most similar to all of the other gels in the project and designated that gel as the master gel to which all other images were matched. The log-standardized abundance was the variable subjected to statistical analysis. The standardized abundance was derived from the normalized spot volume (with background subtracted) and standardized against the intra-gel standard. The log of standardized abundance values were used so that data points approached a normal distribution around zero and were therefore suitable for statistical analysis. To examine cardiac protein expression differences in treated and untreated mice, we used a Student’s t-test. The null hypothesis was that there was no change in protein expression and the average ratio (log-standardized abundance) between the two groups being compared was 1. Protein abundance was considered to be significantly different if the t-test p value was < 0.05.

Analysis with SameSpots revealed that 65 spots exhibited a statistically significant average change of at least 3-fold when comparing treated vs untreated (Table 1) samples. These spots were cored from the preparative gel (Figure 2), digested and run on the LTQ mass spectrometer to determine peptide composition. The resulting peptides from each spot were searched against all Mus musculus proteins in the Swiss Prot database (16,665 sequences) using Mascot Daemon to identify candidate proteins responsible for
expression changes. Proteins identifications from selected spots are included in Table 1. Positive values for fold-change indicate greater expression in the samples from the treated group.
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