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Mechanistic and Translational Studies on Skeletal Malignancies

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Dedication:

I am dedicating this dissertation to my grandmother Jean McGuire (1932-2009)
Acknowledgments:

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Abstract:

New treatment strategies are desperately needed for treating skeletal malignancy. Skeletal malignancies can be either primary cancer that originated in the bone, such as osteosarcoma, or metastatic cancer that spread from another organ to the skeleton, as in the case of breast or prostate cancer. In this thesis, I will detail two projects that focus on the discovery of new treatment strategies for both primary skeletal malignancy and metastatic skeletal malignancy.

The first project focuses on the primary skeletal malignancy, osteosarcoma, a rare cancer that is commonly diagnosed in children and young adults and metastasizes to the lungs. The survival rate for lung metastatic patients is dismal and has not improved in the decades since the approval of combination chemotherapeutics for treatment. Our recent work shows that targeting the epigenetic changes is effective in treating osteosarcoma as well as the resulting lung metastases. Using preclinical mouse models, using FDA approved pan-histone deacetylase (HDAC) inhibitors panobinostat and romidepsin we can significantly reduce the growth of primary osteosarcoma the resultant lung metastases as well as prevent the formation of these metastases. We propose that HDAC inhibition of HDACs could be effective in treating patients with primary and lung metastatic osteosarcoma.

The second project focuses on metastatic prostate cancer. By investigating the interactions of bone resident mesenchymal stem cells (MSCs) and prostate cancer cells, we discovered that secreted factors from the MSCs, including interleukin 28 (IL-
28), promote the evolution of apoptotic resistant prostate cancer cells. The signal transducer and activator of transcription proteins (STAT) signaling pathway in these MSC educated prostate cancer cells becomes altered making them sensitive to STAT3 inhibition. Treatment both *in vitro* and *in vivo* with the small molecule STAT3 inhibitor, S3I-201 effectively kills the apoptotic resistant prostate cancer cells. We propose that prostate cancer cells are selected by MSCs in the bone microenvironment to become resistant to chemotherapies (e.g., docetaxel) while at the same time become more sensitive to STAT3 inhibition. Based on these findings, we believe that targeting STAT3 signaling is a therapeutic option for men with incurable bone metastatic prostate cancer.
Chapter 1: Cancer and Metastasis of the Skeleton

Cancer Overview:

Cancer affects nearly everyone at some point, be it by personal diagnosis or by the diagnosis of a friend or family member. In 2020, 1.8 million new cancer diagnoses and over 600,000 deaths are estimated in the United States [1]. Cancer occurs when abnormal cell proliferation creates a mass of cells called a tumor. Tumors can be benign or malignant. Benign tumors are non-cancerous, meaning that they do not have the ability to invade local tissue and spread to other parts of the body. However, they can still be dangerous depending on their size and the area of the body that they form. Malignant tumors are always dangerous and can lead to fatalities due to their ability to metastasize, or spread, to other tissues and organs.

The multifaceted complexity of cancer cells was summarized in 2000 by Robert Weinberg and Douglass Hanahan as the “hallmarks of cancer.” Six hallmarks were initially proposed, with 2 additional hallmarks, and 2 enabling characteristics that lead to development of “hallmarks” being added in 2011 as our understanding of the disease continues to develop [2, 3]. These hallmarks help to describe what changes are necessary for a normal cell to become a cancer cell. These changes are simplistically described in (Table 1). One hallmark, deregulated metabolism, which was observed by Otto Warburg and called the “Warburg effect,” led to a Nobel prize. Normal cells use oxidative phosphorylation for energy production, but cancer cells switch to using a less
efficient form of metabolism know as glycolysis. Otto Warburg hypothesized that this metabolomic change was actually a cause of cancer [4, 5]. Despite extensive knowledge about carcinogenesis, we still have much to learn about the biology of cancer cells and how they interact with other cell types to develop the most insidious aspects of the disease such as metastasis. Approximately 90% of cancer deaths are due to metastatic progression [6], therefore it is of utmost importance to focus on understanding and developing new treatment options for patients with metastatic disease [7].

**Table 1**: The hallmarks of cancer and how each hallmark changes a normal cell to become a cancer cell.

<table>
<thead>
<tr>
<th>Hallmarks of Cancer</th>
<th>Normal Cells vs Cancer Cells</th>
</tr>
</thead>
</table>
| Self Sufficiency in growth signals | Normal cells need a stimulus to proliferate  
Cancer cells proliferate without any stimulus |
| Insensitivity to antigrowth signals | Normal cells react to surrounding cells halting their proliferation  
Cancer Cells ignore signals from the surrounding cells |
| Evading programmed cell death | Normal cells will die when they become damaged or infected  
Cancer Cells develop multiple ways to avoid apoptosis |
| Limitless replicative potential | Normal Cells will divide a finite number of times  
Cancer Cells can continue to divide infinitely |
| Sustained Angiogenesis | Normal Cells can not grow without nutrients from blood flow  
Cancer Cells stimulate formation of blood vessels to bring nutrients |
| Tissue invasion and metastasis | Normal cells do not invade surrounding tissue or colonize new tissue  
Cancer cells gain the ability to invade new areas of the body |
| Deregulated metabolism | Normal cells often use oxidative phosphorylation to create energy  
Cancer cells use glycolysis to create energy |
| Evading the immune system | Normal cells are not detected by the immune system  
Cancer cells can cloak themselves so they are not recognized as abnormal by the immune system |
| Genome instability | Normal cells have a diploid number of 46 chromosomes  
Cancer Cells typically develop an abnormal chromosome number |
| Inflammation | Chronic inflammation can cause normal cells to become cancer cells |
Metastasis Overview:

The process of a primary tumor relocating and establishing in a distant site is known as metastasis. The metastatic cascade is a multistep process that begins with the primary tumor invading the local stroma and intravasating either the blood circulatory system and/or the lymphatic system (Figure 1) Most solid tumors are epithelial in origin and do not readily invade and migrate. It has been proposed that epithelial tumor cells undergo an epithelial to mesenchymal transition (EMT) to acquire traits that promote migration and invasion into the underlying basement membrane where they can gain access to vasculature. A key protein involved in the process is E-cadherin. E-cadherin is recognized as a tumor suppressor gene that functions in maintaining cell-to-cell contact/adhesion of epithelial cells. It has been demonstrated both in vitro and in vivo using murine models that expression of E-cadherin can suppress invasion and metastasis [8, 9]. In metastatic cancer cells, however, expression of E-cadherin is lost, whereas expression of N-cadherin is increased [10]. This in turn can promote invasion and metastasis. In breast cancer cells, expression of N-cadherin promotes adhesion to endothelial cells and interaction with fibroblast growth factor receptor (FGFR). During stimulation with fibroblast growth factor (FGF), these cells upregulate matrix metalloproteases (MMPs), specifically MMP-9, causing the breast cancer cells to become more invasive and metastatic [11] [12]. In prostate cancer, N-cadherin expression is correlated with castration resistance, and an in vivo study treatment with an antibody to block the extracellular domain of N-cadherin significantly inhibited prostate cancer proliferation and metastasis [13]. Once the cancer cells have successfully invaded the local stroma, they can intravasate into either the blood stream
or lymphatic system [14]. The loss of contact with other cells and extracellular matrix normally triggers a type of cell death called anoikis. To avoid anoikis, cancer cells commonly upregulate genes for anti-apoptotic proteins such as cellular FLICE-like inhibitory protein (c-FLIP) and B-cell lymphoma 2 (BCL-2) family proteins [15]. To survive the circulating tumor cells must evade immune detection and/or create an immunosuppressive environment while traveling through the body [16]. Cancer cells must then extravasate at distant sites. At this point they can remain dormant sometimes for decades, before they eventually begin to proliferate and create a secondary tumor [17].

**Figure 1**: An illustration of the five major steps in the metastatic cascade.
Interestingly, certain primary tumor types have a predilection to colonize specific secondary organs, as is the case of prostate cancer metastasizing to the skeleton. In 1889 Stephen Paget proposed the “seed and soil” theory, after studying autopsy records of breast cancer patients. His theory states that a metastatic site is determined by interactions between “seeds”, (the cancer cells), and “soil”, (the microenvironment of metastatic site). He proposed that the seeds will only establish in fertile soil, meaning that the metastatic site is not random [18]. This theory was challenged by James Ewing in 1928 who proposed that metastasis is determined by random dispersion through the circulatory or lymphatic system and therefore is determined by anatomy [19]. Nearly 50 years later, the validity of both theories was confirmed by a series of experiments by Josh Fidler, who demonstrated that melanoma cells preferentially colonized the lung. When radio-labeled melanoma were injected intravenously into mice, they distributed to all organs, supporting the Ewing hypothesis, but they would only survive and grow in the lung, supporting the Paget hypothesis [20]. When lung and renal tissues were implanted into the muscle tissue of mice injected with melanoma cells, metastatic outgrowth only occurred in the implanted lung tissue and the lungs of the mice [21]. Therefore, different metastatic sites have “fertile soil” for different “seeds,” or cancer types. To better understand why certain cancers, develop and/or metastasize to bone, it is essential to understand the biology and environment of the skeleton.

The Human Skeleton:

The skeleton is most commonly thought of as the structural support for the body and a source of protection for our internal organs. However, the skeleton is actually a much
more complex, multi-functional organ. For example, it acts as the storage facility of calcium and houses the bone marrow, where important stem cell populations of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) reside [22]. Hematopoietic stem cells differentiate into the different blood cells and MSCs differentiate into, adipose, cartilage, muscle- and bone-forming cells [23, 24].

Type 1 collagen constitutes approximately 95% of the bone [25]. Whereas non-collagenous proteins and proteoglycans make up the rest of the bone matrix. A calcium containing compound hydroxyapatite, (CA\(_{10}\)[PO\(_4\)]\(_6\)[OH]\(_2\)), is responsible for mineralizing and hardening the matrix of the bone, which is composed of non-mineralized type-1 collagen or osteoid. The osteoid is produced by the bone forming cells, osteoblasts [26, 27]. Mineralized bone contains many different growth factors that are held in reserve. These trapped growth factors such as transforming growth factor beta (TGF\(\beta\)), bone morphogenic proteins (BMPs), insulin-like growth factors (IGFs), and others can be released upon bone resorption [28]. The body has five types of bones long, flat, short, sesamoid and irregular. Long bones include but are not limited to the tibia, femur, fibula, humorous, radius and ulna. Short bones are bones that mediate little to no movement and include the small bones in the hands and feet, carpals and tarsals, respectively. Flat bones function as protection and include the cranium and rib cage. Sesamoid bones are bones that are surrounded by tendon or muscle, the knee cap is the largest of these bones. Irregular bones are the rest of the bones that can not be classified into the other four groups. The vertebra in the spinal column are an example of irregular bones [29]. Bones consist of two different bone types, cortical and trabecular or cancellous bone. Cortical bone is the hard-dense exterior of many bones
including the long bones that account for approximately 80% of the total skeletal mass. Cortical bone that appear solid are actually porous and allows blood flow and nerve fibers to innervate the bone [30]. The second type of bone is called trabecular or cancellous bone, and is found within the bone marrow at the ends of long bones, as well as in flat and irregular bones. Trabecular bone is less dense and more metabolically active, contributing to the maintenance of HSCs and MSCs [31].

**Skeletal Malignancy:**

Malignant bone tumors can be categorized into primary, originating in the bone, or metastatic, tumors that arise in other organs and subsequently colonize the bone. It should be noted that there are hematological malignancies such as multiple myeloma and leukemias that originate in and colonize the bone as well, but they will not be the focus of this thesis since they were not studied in any of our experiments. Primary bone cancer comprises a small percentage of cancer diagnoses. In the United States, it is estimated that approximately 3,500 new cases of bone cancer will be diagnosed in 2020 [32]. Metastatic bone cancer is more common, with prostate being the most prevalent. Approximately 25% of prostate cancer patients develop bone metastases, and these patients will have a 5-year survival rate of only 3% [33]. Other cancers have a tendency to metastasize to the skeleton, including lung, kidney, breast and gastrointestinal. The percentage of patients who develop bone metastases with these cancers are 12, 8, 6, and 3% respectively [34].
Chapter 2: Primary Skeletal Malignancy and Osteosarcoma

Primary Skeletal Malignancies Overview:

Primary bone cancers include osteosarcoma, chondrosarcoma, fibrosarcoma, giant cell tumor of the bone and Ewing’s sarcoma. This thesis will focus on treatment primary and metastatic osteosarcoma; therefore, osteosarcoma and the resultant metastases will be described in greater detail after the brief overview of other primary bone cancers.

Chondrosarcoma, Fibrosarcoma, Giant Cell Tumor and Ewing’s Sarcoma:

Chondrosarcoma is a cancer of the cells that form cartilage. The most common bones affected are the pelvis and other long bones such as the femur, humerus and ribs [35]. The lung is most common site for metastasis with 50-70% of high-grade chondrosarcoma having evidence of lung metastasis [36]. Fibrosarcoma is a cancer of the fibroblasts, or cells that form fibrous connective tissue. Fibrosarcoma commonly forms in deep soft tissue or periosteum, the membrane that surrounds the bones. It can also arise inside bones in the medullary canal, or bone marrow cavity, as either a primary tumor or metastasis [37]. Giant cell tumor of the bone (GCTOB) also known as osteocalstoma is an uncommon tumor that is pathologically diagnosed by large multinucleated osteoclast type cells. GCTOB is rarely malignant, but in approximately 2% of cases, it metastasizes to the lung [38, 39].
Ewing’s sarcoma is a tumor that can grow in the soft tissue surrounding the bones or in the bone. Primary bone cancers can often be cured with amputation and radiation if diagnosed before metastasis; if there is evidence of metastasis, the survival rates drop significantly [40]. This thesis will focus on treatment of primary and metastatic osteosarcoma so it will be described next in detail.

**Osteosarcoma:**

Osteosarcoma, the most common primary malignancy of bone, is a tumor of mesenchymal origin, giving rise to malignant osteoblast-like cells that are characterized by malignant osteoid deposition [41]. Osteosarcoma accounts for approximately 1000 new cases each year [32]. The lifetime incidence of osteosarcoma has a bimodal distribution with a peak among adolescents and a smaller peak after the age of 70, thought to be secondary to long-standing Paget’s disease and radiation [42]. Over 85% of osteosarcomas are considered primary bone cancers, a de novo bone malignancy of unknown etiology [43]. A typical patient presentation is an adolescent with a history of localized pain and swelling near the metaphysis of a long bone with abnormal bone deposition and periosteal reactivity on x-ray [44]. In the pediatric population, osteosarcoma accounts for only 5% of malignancies, but due to a high degree of heterogeneity and a propensity to metastasize, osteosarcoma contributes disproportionately to cancer-related death in children and young adults [45].

Prior to the 1970s, osteosarcoma treatment primarily consisted of local surgical control, normally amputation, with 5-year survival rates of 20% [46] [47]. With the advent of chemotherapies, including high dose methotrexate, doxorubicin and cisplatin
(MAP), ifosfamide and etoposide (IE), bleomycin, and vincristine, survival rates increased to nearly 67% for localized patients. Disappointingly, survival rates have remained at this level for the past three decades despite advances in our understanding of the disease [46, 48, 49]. This is due in part to the fact that osteosarcoma is highly aggressive, exemplified by the fact that approximately 15% of patients will already have clinically detectable metastases at the time of diagnosis [50, 51]. The lung is the major site of osteosarcoma metastasis followed by secondary skeletal sites [52]. Current treatment approaches for patients with lung metastatic disease involves systemic MAP chemotherapy, both surgical resection of the primary along with metastectomy of all visible lesions and adjuvant high-dose chemotherapy but 5-year survival for these patients remains at approximately 20% inversely proportional to the degree of metastatic disease [52-54]. Clearly, there is room for significant improvement in regard to how the disease is tackled both at the primary and metastatic site.

Among the challenges faced in developing new agents to combat the disease is the high degree of osteosarcoma heterogeneity, chromosomal instability, multiple mechanisms of chemoresistance and, the complex interactions between the cancer cells and the surrounding bone microenvironment [55, 56]. Recent advances have clearly defined the path forward, mainly through forging collaborations towards improving the infrastructure of tumor banking, identifying the top scientific areas of inquiry [57], creating a scoring system to assign priorities for agents towards translation based on preclinical data [58], creating international collaborations to identify a standard chemotherapeutic approach [49], and perhaps most importantly, creating statistical rules for earlier phase clinical trials [59]. Moving forward, there is now a clear trial
design and threshold for what would be considered an active regimen for trials in relapsed patients.

**Osteosarcoma Etiology:**
Osteosarcoma encompasses a variety of osteogenic and chondrogenic primary bone tumors. The former is hallmarked by areas of bone formation and can be found within the bone (central), growing from the inner bone lining (periosteal) or on the outer bone surface (parosteal). The lesions are easily identifiable by x-ray due to their dense calcified matrix, but they also contain areas of active osteolysis mediated by mature bone resorbing osteoclasts. Osteogenic osteosarcoma arises from malignant mesenchymal stem cells, osteoblast precursors through inactivation of multiple tumor suppressors although the precise source has been difficult to identify. Studies into genetic events involved in the genesis and progression of osteosarcoma cancer cells have revealed a complex landscape that underscores the challenges in defining and optimizing new therapeutic strategies [55, 56].

**Osteosarcoma Cancer Cell Heterogeneity and Chromosomal Instability:**
There is a large degree of both inter- and intra-tumor heterogeneity in osteosarcoma, and these differences impact prognosis and response to therapy. Histologically, osteosarcoma is diagnosed as a mesenchymal neoplasm producing extracellular matrix proteins and osteoid [43]. This feature gives rise to the main histologic subtypes of osteosarcoma: osteoblastic, chondroblastic, and fibroblastic. A study of 570 patients of the European Osteosarcoma Intergroup demonstrated that, in comparison to the
traditional osteoblastic subtype, there was better response as indicated by the percentage of necrotic tumor present upon resection to standard therapy in the fibroblastic group and a lower proportion of good responders in the chondroblastic group [60]. In addition to histological inter-tumor differences, there is also significant intra-tumor heterogeneity with a large number of subclones per biopsy. The degree to which osteosarcoma exhibits these intratumoral differences has been demonstrated with immunohistochemistry, flow cytometry and genomic studies [61, 62].

The underlying mechanism for this intratumoral diversity appears to be the genetic instability observed in osteosarcoma. Osteosarcoma is characterized by a complex and unstable karyotype with different clones having a unique pattern of complex chromosomal abnormalities. The tumors exhibit gene amplifications, aneuploidy and multiple unbalanced chromosomal rearrangements. However, there is no pathognomonic mutation or translocation such as the Philadelphia chromosome in CML [63]. The majority of the instability is observed at the chromosomal level and multiple studies have demonstrated that osteosarcoma has one of the highest rates of chromosomal mutations, such as structural and copy number variations [63-65]. The chromosomal rearrangements occur potentially via breakage-fusion-bridge (BFB) where uneven breaks during anaphase and cytokinesis result in potentially new chromosomes [63, 66, 67] chromothripsis, a catastrophic event found in 33% of osteosarcomas in which chromosomes shatter into multiple pieces and are incorrectly, non-homologously “stitched” back together [68] and; alternative lengthening of telomeres (ALT), a process that can result in rapid changes to telomere length and produces free ends that could give rise to end-end interactions that can feed the BFB cycle [69]. ALT has been
observed in 85% of osteosarcoma and is associated with poorer outcomes [55, 70]. Underpinning these chromosomal rearrangement mechanisms is a frequent amplification of DNA helicases. For example, a gain of function in the RECQL DNA helicase, RECQL4, has been identified in 33% of osteosarcoma patients [71, 72]. RECQL4 is important in DNA replication and repair and aberrant expression has been shown to highly correlate with a risk for osteosarcoma development. Clearly, osteosarcoma progression is hallmarked by aberrations in DNA replication/repair making this area an important angle of therapeutic attack.

**Targeting Osteosarcoma DNA Replication and Nuclear Function:**
Classic chemotherapies such as doxorubicin and etoposide are geared toward disrupting DNA replication. Emerging data highlight epigenetic strategies in the treatment of osteosarcoma. Histone deacetylases (HDACs) are critical for regulating gene expression and play an important role in cell proliferation. Inhibition of HDACs can lead to cell cycle arrest, apoptosis and, in some cases, differentiation. Panobinostat, a broad spectrum HDAC inhibitor, has been approved for use in multiple myeloma, and a number of pre-clinical studies have shown efficacy for HDAC inhibitors against osteosarcoma either as a single agent or in combination with other therapies [73-75]. Recently, a combination of panobinostat with the proteasome inhibitor, carfilzomib has been shown to be highly synergistic in promoting apoptosis of multiple osteosarcoma cell lines [76]. These data indicate a promising role for HDAC inhibitors for the treatment of osteosarcoma provide strong preliminary data for the generation of clinical trials in this area.
What are Histone Deacetylases (HDACs)?

HDACs are a group of enzymes that have the ability to remove acetyl groups from histones and other proteins. The examination of calf thymus extract in 1969 led to the discovery of these enzymes [77]. Deacetylation of histones changes the nucleosomal conformation, making the chromatin inaccessible to transcription factors, in turn silencing genes [78]. This epigenetic gene silencing effect is reversed by a group of enzymes called histone acetyltransferases (HATs) that acetylate histones, opening the chromatin and allowing transcription factors access to their promoters [79]. Many non-histone proteins can become deacetylated by HDACS [80]. Nearly 3 decades later in 1996, the first HDAC was isolated and cloned (HDAC1) [81]. HDACs are numbered chronologically by the dates that they were discovered. [82] There are 4 classes of HDACs, including 18 individual HDACs and sirtuins. The sirtuins are named based on their relation to the yeast protein silent information regulation 2 (sir2) they are unrelated to the “classical HDACs” but they are included as the third class of HDACs due to the fact that they are NAD-dependent deacetylase enzymes [83]. NAD-dependent Class I includes HDAC1, HDAC2, HDAC3, and HDAC8; class II include HDAC4, HDAC5, HDAC6, HDAC7 and HDAC9; Class III are the sirtuins and include SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7; class IV solely consists of HDAC11 due to the fact that it is structurally distinct from the other HDACs [84].
**HDACs Role in Cancer Progression:**

HDACs play numerous roles in cancer progression and metastasis by de-acetylating histones and non-histone proteins. HDACs regulate many cellular processes including but not limited to cell cycle, autophagy, angiogenesis, apoptosis and DNA damage response [84]. HDAC inhibition has been shown to cause cell cycle arrest in G₁/S or G₂/M phase [85]. Overexpression of HDACs has been implicated in poor prognosis in liver, lung, breast, pancreatic, prostate, colorectal and gastric cancers. [86-92] On the other hand, inactivation of certain HDACs has been shown to be oncogenic. For example, poor prognosis in lung cancer patients is associated with low expression of class II HDACs, particularly HDAC 10 [87]. In hepatocellular carcinoma (HCC) low expression of HDAC 6 is a poor prognostic factor for patients that had a liver transplant. Experimental HDAC 6 knockdown caused an increase in angiogenesis by upregulation of VEGFA, and also suppressed HCC apoptosis [93].

**HDAC Inhibitors for Cancer Treatment:**

HDAC inhibitors are a group of chemical compounds that are divided into 5 different groups based on their chemical structure the include hydroxamates, short-chain fatty acids, cyclic tetrapeptides, benzamides and most recently hydrazides [94, 95]. Hydroxamates were the first group of HDAC inhibitors synthesized. They include, belinostat, tricostatin A, tubacin, vorinostat and panobinostat among others. Of the hydroxamates, vorinostat was the first FDA approved HDAC inhibitor in 2006 for cutaneous T-cell lymphoma (CTCL). Nearly a decade later, in 2015, another HDAC inhibitor, panobinostat was FDA approved for the treatment of multiple myeloma [96].
Short chain fatty acid HDAC inhibitors include butyrate and valproic acid (VPA). VPA was FDA approved for epilepsy treatment in 1978 and has been shown to exhibit anticancer properties as a HDAC inhibitor in many cancers breast, prostate osteosarcoma, and non-small cell lung cancer [99] [100-103]. Cyclic tetrapeptide HDAC inhibitors include apicidin, trapoxin A and romidepsin (FK228). Romidepsin was FDA approved in 2009 for the treatment of CTCL [104]. Benzamide HDAC inhibitors include SNDX275, MGCK0103 and entinostat (MS-275) of these entinostat was used in clinical trials but was not effective when compared to vorinostat for multiple reasons including pharmacokinetic issues and off target toxicities [105]. The newest group of HDAC inhibitors, hydrazides, are being developed to selectively inhibit class I HDACs with a focus on inhibiting specifically HDAC 3 for the treatment of acute myeloid leukemia (AML) [95].

Although all of the currently FDA-approved HDAC inhibitors are used to treat hematological malignancies, a growing body of preclinical and clinical data showing that HDAC inhibition can be effectively used to treat solid malignancies as well. Two different phase 1 clinical trials testing HDACi for advanced solid malignancies reported a complete response using belinostat and a partial response using panobinostat to treat advanced urothelial carcinoma. In both studies of the patients had mutations in ARID1A, suggesting that patients with these mutations could benefit from treatment with HDACi [106]. In vitro studies in osteosarcoma cell lines have shown that HDAC inhibitors such as valproic acid can impair oxidative phosphorylation, cytoskeleton remodeling, cell cycle, angiogenesis and ubiquitin proteasome pathways [107, 108].
Treatment with vorinostat, which inhibits Class I, II and IV HDACs, can impair the \textit{in vitro} invasive potential of murine osteosarcoma cell lines [109].
Chapter 3: Histone Deacetylase Inhibition Prevents the Growth of Primary and Metastatic Osteosarcoma

Rationale:

Despite being the most prevalent primary bone cancer, the treatment paradigm for osteosarcoma, consisting of high-dose methotrexate, doxorubicin, and cisplatin, has changed little over the past 3 decades [110, 111]. Metastasis is the cause of osteosarcoma related deaths, with the lungs being the most prevalent site [112]. Tragically, the 5-year event-free survival for patients with lung involvement is only 28% [113]. Amongst the provocative questions created by the Osteosarcoma Biology Committee were an improved understanding of metastasis and alterations in copy number, aneuploidy and epigenetic control of osteosarcoma pathogenesis [114]. Due to the rarity of the disease, clinical trials are difficult and time consuming to conduct, underscoring the need for strong preclinical data to inform human trial design. To this end, we assessed the efficacy of approved single agents and combinations using 5 common human osteosarcoma cell lines, focusing on reported tolerability in clinical trials, non-overlapping toxicities, pharmacokinetic data, cytochrome P450 metabolism, and other characteristics that negate drug-drug interactions [115]. Our in vitro data consistently indicated that the broad spectrum HDAC inhibitor, panobinostat, was cytotoxic for osteosarcoma cells at very low nanomolar concentrations, a finding supported by independent studies [115].

Portions of this chapter have been previously published and are utilized with permission of the publisher. (Appendix B p152)
Panobinostat inhibits all classes of HDACs with varying potency and is currently approved for use in multiple myeloma patients with ongoing clinical trials for other malignancies [97]. The primary mechanism of action of panobinostat is disrupting epigenetic control of transcriptional programs that govern cell growth and survival [116]. However, independent of epigenetic regulation, HDACs can also regulate the activity of several proteins involved in the cell cycle, the production of reactive oxygen species, apoptosis, immune surveillance, angiogenesis and tumor metastasis [117].

In vitro studies in osteosarcoma cell lines have shown that HDAC inhibitors such as valproic acid can impair oxidative phosphorylation, cytoskeleton remodeling, cell cycle, angiogenesis and ubiquitin proteasome pathways [107, 108]. Treatment with vorinostat, which inhibits Class I, II and IV HDACs can impair the in vitro invasive potential of murine osteosarcoma cell lines [109]. Further, osteosarcoma cell lines are highly sensitive to panobinostat treatment at nanomolar concentrations [115]. Here, we report, using in vivo models of osteosarcoma, that panobinostat as a single agent is highly effective at preventing orthotopic osteosarcoma growth, spontaneous lung metastasis, lung colonization by osteosarcoma cell lines and the outgrowth of established osteosarcoma lung metastases. Additionally, genetic and pharmacological studies establish key roles of HDAC1 and HDAC2 in controlling the progression of the disease.

Results:

Panobinostat prevents the growth of orthotopic osteosarcoma:
We initially assessed the activity of panobinostat in 3 in vivo models of osteosarcoma; murine K7M2 [118], and human SAOS2 and lung metastatic derived SAOS2-LM7
osteosarcoma cell lines [119] that can be orthotopically or tail vein inoculated as models of primary and metastatic disease. qRT-PCR and immunoblot results revealed that several HDACs were expressed in these osteosarcoma cell lines (Figure 2 a and Figure 5 a). Analysis of the St. Jude PeCan database also revealed the expression of several HDACs in human osteosarcoma patients (n = 107; Figure 2 b) [120]. In vitro, dose-response studies on osteosarcoma cell lines established that panobinostat had a similar IC₅₀ (14.3 nM) in K7M2, SAOS2 (11.98 nM) and SAOS2-LM7 (25 nM) Figure 5 b). As expected, panobinostat treatment of all 3 cell lines led to increased levels of in acetylated histone H3 (Figure 5 c).

To initially test the efficacy of panobinostat in vivo we utilized the syngeneic K7M2 murine osteosarcoma transplant model in immunocompetent Balb/c mice [118]. Mice were intratibially injected with luciferase-expressing K7M2 cells and contralateral limbs received control injections of saline. Using bioluminescence as a correlate for tumor growth, mice were randomized into vehicle control (VEH: n=11) or panobinostat (PANO: n=11) groups. Panobinostat treatment inhibited or significantly delayed primary osteosarcoma growth (Figure 5 d), where analysis of the average bioluminescence reveals significant differences between the groups from day 14 onwards (Figure 5 e). Of note, panobinostat-treated mice were given a drug holiday between days 23-30 due to diarrhea and anemia that mitigated the observed toxicities but resulted in osteosarcoma growth. However, upon resuming panobinostat treatment, the disease burden again plateaued.
Figure 2: HDAC expression in osteosarcoma cell lines and osteosarcoma patient specimens.

a. qRT-PCR analysis of HDAC gene expression in human and mouse osteosarcoma cell lines. Gene expression for each HDAC examined target is normalized to the actin levels for each cell line and graphed as $2^{-\Delta\text{ACT}}$. b. Analysis of HDAC gene expression (RNA-Seq) osteosarcoma (OS) patients (n=107). Data is shown fragments per kilobase million (FPKM) and generated from the St. Jude PeCan database ([https://pecan.stjude.cloud/proteinpaint/](https://pecan.stjude.cloud/proteinpaint/)) [120].
Using a bioluminescence value of $1 \times 10^6$ as the study endpoint, the median progression time of 21 days for the control cohort was significantly shorter than the 53-day median for the panobinostat-treated cohort. Treatment was halted on day 56. At that time point, only 54% of the panobinostat-treated mice ($n = 6/11$) had reached an RLU of $1 \times 10^6$ (Figure 5 f). Upon reaching endpoint, *ex vivo* X-ray analysis revealed a significant increase in osteosarcoma induced osteolysis in the vehicle control versus the panobinostat-treated cohort (Figure 5 g). This was reflected in greater trabecular bone volumes as measured by high-resolution μCT scan analysis although statistical significance was not reached (Figure 5 h). Histomorphometry and histological analyses also revealed no striking differences between the groups (data not shown). However, consistent with bioluminescence data, we observed that panobinostat significantly reduced tumor proliferation and enhanced apoptosis indices as measured by phospho-histone H3 (pHH3) and cleaved caspase 3 (CC3), respectively (Figure 5 i, j).

**Figure 3-1:** Panobinostat treatment impairs primary osteosarcoma growth and associated bone disease.

a. Analysis of HDAC expression in the K7M2, SAOS2 and SAOS2-LM7 osteosarcoma cell lines. Actin (representative image) was used as a loading control. Primary mouse mesenchymal stem cells (MSC) were used as a positive control. Numbers indicate molecular weight (kDa). b. K7M2, SAOS2 and SAOS2-LM7 cell lines were incubated with the indicated concentrations of panobinostat for 24 hours. Cell viability (CellTiter blue) is presented as a percentage of vehicle control.
Figure 4-2: Panobinostat treatment impairs primary osteosarcoma growth and associated bone disease.

c. Acetylated Histone H3 (AHH3; red) immunofluorescent staining in vehicle control (VEH) and panobinostat (PANO: 40nM) treated K7M2, SAOS2 and SAOS2-LM7 cells after 16 hr. DAPI (blue) was used a nuclear stain. d. Spider plot of K7M2 bioluminescence (RLU) over time in individual vehicle control (VEH; n=11) and panobinostat (PANO; n=11) treated mice. Treatment was initiated 3 days subsequent to inoculation (blue arrow). Mice were removed from study upon reaching 1x10^6 relative light.
units (RLU). Representative images show bioluminescence in each group at day 35 with hotter colors indicating greater tumor burden. e. Average of bioluminescence in vehicle control and treated mice. c. Kaplan-Meier curve of time to reach clinical endpoint (RLU=1x10^6) for orthotopic K7M2 cells in the panobinostat (PANO) and vehicle control (VEH) groups. f. X-ray analysis of tumor induced osteolysis (arrow) in vehicle control (VEH) and panobinostat (PANO) treated groups. Tumor induced osteolytic area (TuV) was measured as a function of the total volume (TV) of the marrow cavity. g. μCT analysis of trabecular bone volume in vehicle control (VEH) and panobinostat (PANO) treated groups. Trabecular bone volume (BV) was measured as a function of the total volume (TV) of the marrow cavity.

**Figure 5-3:** Panobinostat treatment impairs primary osteosarcoma growth and associated bone disease.

h, i. *Ex vivo* analyses from study endpoint of proliferative and apoptotic indices using phospho-histone H3 (pHH3; red arrows; h) and cleaved caspase 3 (CC3; red, arrows, i) respectively. Scale bars represent 50μm. Asterisks denotes statistical significance (**p<0.01; n.s. denotes non-significance).

**Panobinostat treatment impairs spontaneous lung metastasis:**

The K7M2 model spontaneously metastasizes to the lung from the orthotopic site at high frequency [118]. In the vehicle cohort, we observed the median time to detectable lung metastasis, assessed by the appearance of bioluminescent signal, was 10 days versus 21 days for the panobinostat-treated mice (Fig 4a). Of note, 27% of the panobinostat treated cohort (n=3/11) had no evidence of lung metastasis at the study endpoint (day 56). Further, lung metastases that did form in panobinostat-treated
recipient mice were significantly smaller than those formed in the vehicle group (Fig. 4b). In accord with this observation, the number of tumor nodules in hematoxylin and eosin (H&E) stained sections were significantly reduced in tumor bearing lungs obtained from panobinostat-treated versus vehicle-treated mice (Fig. 4c). The proliferative indices, as measured by pHH3, were also significantly lower in the panobinostat-treated cohort. Conversely, apoptotic indices (CC3) were significantly higher compared to vehicle control (Fig. 4d, e). Thus, panobinostat is a potent inhibitor of primary osteosarcoma growth and metastasis in this aggressive in vivo immunocompetent model of the disease.

**Pretreatment with panobinostat suppresses osteosarcoma lung seeding:**

Given that panobinostat treatment reduced spontaneous osteosarcoma lung metastasis, we next tested if panobinostat would prevent the distal seeding and establishment of osteosarcoma in a tail vein pulmonary metastasis model. The purpose of this in vivo experiment was to determine whether panobinostat would potentially be effective clinically as a neo-adjuvant or adjuvant therapy post-primary tumor resection in preventing the seeding of lung with osteosarcoma metastases. Mice were divided into vehicle (VEH: n=9) or panobinostat (PANO: n=5) cohorts and treated for 5 days prior to the tail vein inoculation with K7M2 luciferase-expressing cells.
Figure 6: Panobinostat treatment suppresses spontaneous osteosarcoma lung metastasis.

a. Kaplan Meier curve of time to progression (TTP) of lung bioluminescence detection in mice bearing primary (tibial) K7M2 tumors in the vehicle control (VEH; n=11/11) group and panobinostat (PANO; n=8/11) treated groups. b. Analysis of bioluminescence (AVG. RLU) in spontaneous metastases arising from orthotopic primary K7M2 tumors in the vehicle control (VEH; n=11/11) group and panobinostat (PANO; n=8/11) treated groups. c. Tumor volume of lung metastases (arrows) as a percentage of total volume in H&E stained sections derived from vehicle control (VEH) and panobinostat (PANO) treated mice. Arrows indicate metastatic lesions. d, e. Ex vivo analyses from study endpoint of proliferative and apoptotic indices using phospho-histone H3 (pHH3; red arrows; d) and cleaved caspase 3 (CC3; red, arrows, e) respectively. Scale bars represent 50μm. Asterisks denotes statistical significance (*p<0.05, **p<0.01; ****p<0.0001; n.s. denotes non-significance).

Bioluminescent analysis revealed an initial drop in both groups after inoculation but subsequent to engraftment, lower tumor burden and growth rates were observed in the lungs of the panobinostat-treated mice (Figure 7 a). Analysis of the average
bioluminescence revealed significant differences between the groups from day 27 onwards (Figure 7 b). Panobinostat treatment was halted at day 38 post tumor inoculation as statistical significance had been reached. Disease progression, using the clinical endpoint of labored breathing or weight loss, was then monitored in the remaining mice through day 92. At day 74, all control mice had succumbed to lung metastasis with a median survival time of 54 days (Figure 7 c). In contrast, only 60% (n=3/5) of the mice treated with panobinostat for 38 days had succumbed at the study endpoint and the median survival time was significantly higher at 92 days versus the vehicle cohort.

Detailed analyses of lung metastases revealed far fewer gross surface metastases in the panobinostat-treated group (Figure 7 d) as well as fewer micro metastases (Figure 7 e). The metastases were distinct, and regardless of group, did not display striking differences in morphology or location within the lung tissues. Interestingly, despite ceasing panobinostat treatment at day 38, proliferative (pHH3) and apoptotic (CC3) indices remained significantly lower and higher, respectively, in the panobinostat treated cohort at the study endpoint (Figure 7 f, g). Thus, panobinostat-treatment is effective at limiting the colonization of the lung by osteosarcoma cells.

**Panobinostat treatment reduces established osteosarcoma lung metastatic burden:**

In pretreatment seeding studies, 40% (n = 2/5) of the panobinostat-treated mice had detectable tumor burden but remained viable at the study endpoint (day 97) despite ceasing treatment at day 38.
Figure 7: Panobinostat prevents seeding of lung metastatic osteosarcoma.

a. Spider plot of K7M2 bioluminescence (RLU) over time in individual pre-treated vehicle control (VEH; n=9) and panobinostat (PANO; n=5) treated mice. Treatment was initiated five days prior to tail vein inoculation of the osteosarcoma cells (blue arrow). Representative images show bioluminescence in each group at day 35.

b. Average of bioluminescence in vehicle control and treated mice.

c. Kaplan-Meier curve of time to reach clinical endpoint in the panobinostat (PANO) and vehicle control (VEH) groups. Panobinostat treatment was stopped at day 38 (red arrow) once statistical significance in overall survival had been reached. At day 97, all remaining mice in the panobinostat group were euthanatized (n=2).

d. Lungs from each group were inflated with formalin upon removal and the number of surface metastases (Metastases #) counted.

e. Lung sections derived from each group were stained with hematoxylin and eosin and the percent tumor volume as a function of total lung volume calculated.

f, g. Ex vivo analyses from study endpoint of proliferative and apoptotic indices using phospho-histone H3 (pHH3; red arrows; f) and cleaved caspase 3 (CC3; red, arrows, g) respectively. Scale bars represent 50μm. Asterisks denotes statistical significance (*p<0.05; **p<0.01; ***p<0.001).
To assess if the outgrowing established metastases remained sensitive to panobinostat, a single mouse from this cohort was re-treated with panobinostat at day 90. Within 1 week, the tumor volume, as measured by bioluminescence, was reduced by approximately 75% (Figure 8). Further, K7M2 metastatic cells isolated from the lungs of panobinostat-treated mice, which were selected in culture using puromycin (selection marker for luciferase expressing cells) containing media, remained sensitive to low nM doses of panobinostat (Figure 8). Thus, these osteosarcoma cell lines did not acquire resistance to the HDAC inhibitor in vivo.

![Figure 8: Panobinostat retreatment reduces outgrowing lung metastatic osteosarcoma.](image)

**a-d.** Bioluminescent (RLU) analysis of K7M2 lung metastatic growth in mouse #23 treated with panobinostat until day 38 a, when treatment ceased. At day 90, panobinostat treatment was reinitiated b, with subsequent imaging at day 97 c. Bioluminescent (RLU) values are shown for each point d. Treatment cessation and initiation are indicated by red and blue arrows respectively. e. Metastatic K7M2 cells isolated from lung tissue were treated with Panobinostat over 24 hours and the impact on growth (RLU) determined.

Given that panobinostat impaired osteosarcoma lung seeding, we next tested if this drug would also reduce the growth of established lung metastases. K7M2 cells were tail vein inoculated and allowed to establish and grow in the lung. At day 3 post-inoculation, mice were randomized based on bioluminescence into vehicle control, (VEH: n = 12) or panobinostat (PANO: n = 12) cohorts. Within 72 hours of treatment, a significantly lower growth rate was detected in the panobinostat treated group compared
to vehicle control and this difference persisted throughout the remainder of the study (Figure 7 a). Analysis of average bioluminescence revealed significant differences between the cohorts from day 13 onwards (Figure 7 b). Using a clinical endpoint of $1 \times 10^6$ RLU, overall survival was significantly reduced in the vehicle control group (17 days) compared mice treated with panobinostat (44 days) (Figure 7 c). Importantly, while all vehicle-treated recipients reached the study endpoint by day 33, 75% of the panobinostat group (n = 9/12) were below this cut-off at day 45. Moreover, analysis of gross and micrometastases revealed a significantly lower lung metastatic burden in the panobinostat versus the vehicle cohort (Figure 7 d, e). Finally, analysis of proliferative (pHH3) and apoptotic (CC3) indices revealed lower and higher rates, respectively, of osteosarcoma lung metastatic growth in the panobinostat treated mice compared to vehicle control (Figure 7 f, g).

Patients with synchronous or metachronous lung metastatic osteosarcoma have a particularly poor prognosis [52]. We therefore, tested the in vivo efficacy of panobinostat in a model of this disease state, the orthotopic SAOS2-LM7 metastasis model [119]. Again, panobinostat treatment (n = 9) significantly reduced the numbers and growth of SAOS2-LM7 lung metastases versus the vehicle-treated cohort (n = 8; Figure 10). Collectively, these models establish panobinostat as an effective single agent treatment for established lung metastatic osteosarcoma.

**HDAC1 and HDAC2 drive osteosarcoma malignancy:**

qRT-PCR and immunoblot analysis revealed that several HDACs are expressed in the osteosarcoma cell lines models used (Figure 2 a and Figure 5 a).
Figure 9: Panobinostat impairs the growth of established lung metastatic osteosarcoma

a. Spider plot of K7M2 bioluminescence over time in individual vehicle control (VEH; n=12) and panobinostat (PANO; n=12) treated mice. Treatment was initiated three days post inoculation of the osteosarcoma cells (blue arrow). Representative images show bioluminescence in each group at day 35.

b. Average of bioluminescence in vehicle control and treated mice.

c. Kaplan-Meier curve of time to reach clinical endpoint in the panobinostat (PANO) and vehicle control (VEH) groups.

d. Lungs from each group stained for K7M2 expression.
group were inflated with formalin upon removal and the number of surface metastases (Metastases #) counted. e. Lung sections derived from each group were stained with hematoxylin and eosin and the percent tumor volume as a function of total lung volume calculated. f, g. *Ex vivo* analyses from study endpoint of proliferative and apoptotic indices using phospho-histone H3 (pHH3; red arrows; f) and cleaved caspase 3 (CC3; red, arrows, g) respectively. Scale bars represent 50μm. Asterisks denotes statistical significance (*p<0.05; **p<0.01; ***p<0.001).

**Figure 10:** Panobinostat and romidepsin significantly impact the growth of established lung metastatic SAOS-LM7 osteosarcoma.

a, b. Spider plot (a) of SAOS-LM7 bioluminescence over time in individual vehicle control (VEH; n=8) or panobinostat (PANO; n=9) treated mice. Treatment was initiated seven days post inoculation of the osteosarcoma cells. Tumor volumes based on bioluminescence (RLU) were compared at day 147 (b). c, d. Spider plot (a) of SAOS-LM7 bioluminescence over time in individual vehicle control (VEH; n=8) or romidepsin (ROMI; n=9) treated mice. Treatment was initiated 7 days post inoculation of the osteosarcoma cells. Tumor volumes based on bioluminescence (RLU) were compared at day 147 (d). e.
Representative H&E stained lung sections derived from vehicle control, panobinostat and romidepsin treated mice. Asterisks denotes statistical significance (*p<0.05; **p<0.01).

Interestingly, silencing of HDAC5 or HDAC6 significantly increased osteosarcoma growth \textit{in vitro} (Figure 11 a-d), whereas treatment with the HDAC6 selective inhibitor, tubastatin had little impact on osteosarcoma viability with a IC$_{50}$ of only 13.88 μM; i.e., approximately 1000-fold less potent than panobinostat (Figure 11 e). Conversely, silencing of HDAC1 or HDAC2 impaired osteosarcoma cell growth (Figure 12 a, b). Because we noted compensatory expression of HDAC1 upon HDAC2 silencing, we also examined effects of combined HDAC1/2 silencing, which significantly reduced the growth of the osteosarcoma cells (Figure 12 b). Consistent with these findings, K7M2 and SAOS2-LM7 cells were highly sensitive to the HDAC1/2 selective inhibitor, romidepsin with IC$_{50}$ values of 50 nM and 5 nM that were similar to those noted for panobinostat (Figure 12 c, d). As romidepsin inhibits other HDAC family members at higher concentrations [121, 122], we treated HDAC1/2 depleted cells with romidepsin and found no additional effect on osteosarcoma growth demonstrating the selectivity for romidepsin against HDAC1/2 at the concentrations used (Figure 12 e).
Figure 11: HDAC5 and HDAC6 suppress osteosarcoma growth.

a, b. Analysis of HDAC5 levels in K7M2 cells 24 hours subsequent to siRNA silencing (a) compared to scrambled control siRNA (SCR). Actin was used as a positive loading control. Numbers indicate molecular weight (kDa). Densitometry graphs illustrate the impact of silencing on HDAC5 levels. The effect of HDAC5 silencing on cell growth (b) was determined by measuring bioluminescence (RLU).

c, d. Analysis of HDAC6 levels in K7M2 cells 24 hours subsequent to siRNA silencing (c) compared to scrambled control siRNA (SCR). Actin was used as a loading control. Numbers indicate molecular weight (kDa). Densitometry graphs illustrate the impact of silencing on HDAC5 levels. The effect of HDAC6 silencing on cell growth (d) was determined by measuring bioluminescence (RLU).

e. The effects of the HDAC6 selective inhibitor tubastatin at varying concentrations on K7M2 viability over 24 hours.
Figure 12: HDAC1 and HDAC2 contribute to osteosarcoma growth.

a, b. Analysis of HDAC1 and HDAC2 levels in K7M2 cells 48 hours subsequent to siRNA silencing (a) each HDAC individually or in combination (HDAC1/2) compared to scrambled control siRNA (SCR). Actin was used as a positive loading control. Numbers indicate molecular weight (kDa). Densitometry graphs illustrate the impact on HDAC1 and HDAC2 levels. The impact on cell growth subsequent to silencing HDAC1 and HDAC2 or HDAC1/2 (b) was determined by measuring bioluminescence (RLU).

c, d. The effect of the HDAC1/2 selective inhibitor romidepsin at varying concentrations over 48 hours on K7M2 (c) and SAOS2-LM7 (d) cell viability.

e. Romidepsin effects on the viability of HDAC1/2 silenced K7M2 compared to scrambled control (SCR) after 24 hours of treatment. Asterisks denotes statistical significance (*p<0.05; ****p<0.0001; n.s. denotes non-significance).

Given that patients succumb to established lung metastatic osteosarcoma, we examined the efficacy of romidepsin in treating this stage of the disease. To this end, mice were tail vein inoculated with luciferase-expressing K7M2 and, after 3 days, randomized into vehicle (VEH: n=10) or romidepsin treatment cohorts (ROMI: n = 10). Similarly to panobinostat, romidepsin reduced lung metastatic growth as measured by bioluminescence (Figure 14 a) and improved overall survival compared to the vehicle.
cohort (Fig. 10b). Again, reduced growth was associated in lower numbers of gross and micrometastases in romidepsin-treated mice, with an accompanying reduction in proliferation and increased apoptotic indices (Figure 14 c-f). We also observed that romidepsin treatment effectively reduced the growth and number of SAOS2-LM7 lung metastases compared to vehicle (n = 8) (Figure 10 c-e). Based on these data we conclude that HDAC1 and HDAC2 contribute to osteosarcoma progression and metastasis.

**Figure 13-1:** The HDAC1/2 inhibitor romidepsin significantly impacts the growth of established lung metastatic osteosarcoma and the viability of human osteosarcoma PDX cell lines.

**a.** Spider plot of K7M2 bioluminescence over time in individual vehicle control (VEH; n=10) and romidepsin (ROMI; n=10) treated mice. Treatment was initiated three days post inoculation of the osteosarcoma cells. (blue arrow). Representative images show bioluminescence in each group at day 35.

**b.** Kaplan-Meier curve of time to reach clinical endpoint in the romidepsin (ROMI) and vehicle control
(VEH) groups. c. Lungs from each group were inflated with formalin upon removal and the number of surface metastases (Metastases #) counted. d. Lung sections derived from each group were stained with hematoxylin and eosin and the percent tumor volume as a function of total lung volume calculated.

e, f. Ex vivo analyses from study endpoint of proliferative and apoptotic indices using phospho-histone H3 (pHH3; red arrows; e) and cleaved caspase 3 (CC3; red, arrows, f) respectively. Scale bars represent

Figure 14-2: The HDAC1/2 inhibitor romidepsin significantly impacts the growth of established lung metastatic osteosarcoma and the viability of human osteosarcoma PDX cell lines.
50μm. Asterisks denotes statistical significance (*p<0.05; **p<0.01; ***p<0.001). g. Analysis of patient derived xenograft (PDX; SJOS series) cell line response to panobinostat (PANO; blue dots) and romidepsin (ROMI; orange dots). (https://braid.stjude.org/masttour/). 1; CY143B, 2; SAOS2, 3; SAOS2-LM7, 4;SJOS001105_X1, 5;SJOS001107_X1, 6; SJOS001107_X2, 7; SJOS001107_X3, 8; SJOS001108_X1, 9; SJOS001112_X1, 10; SJOS010929_X1, 11; SJOS013769_X1 and 12; U20S. AUC represents area under curve while black dots represent the effectiveness of other reagents.

Osteosarcoma PDX cell lines are sensitive to romidepsin and/or panobinostat:

To determine the robustness of our findings, we examined the sensitivity of patient derived xenograft (PDX) cell lines in a publicly available dataset (https://braid.stjude.org/masttour/) [123, 124]. Consistent with our own current and previous data, analyses reveal a broad sensitivity to romidepsin and/or panobinostat in PDX (n = 8) cell lines (Figure 14 g) [115, 123, 124]. Interestingly, these studies also documented the synergistic effects of HDAC and proteasome inhibitors in vitro. However, in vivo treatment of PDX models with panobinostat and the proteasome inhibitor, bortezomib, did not prove effective compared to controls [123]. Our previous in vitro studies and analysis of K7M2, SAOS-LM7 also documented the synergy between panobinostat and the proteasome inhibitor, carfilzomib (Figure 16)[115]. We next examined the effect of each reagent alone or in combination (at 1/10 of the dose of each single agent based on in vitro data) on the orthotopic growth and spontaneous metastasis of K7M2 in vivo. In contrast to our in vitro data, we observed that, as a single agent, carfilzomib elicited no effect on primary osteosarcoma growth and ultimately did not elicit the beneficial effects observed with panobinostat when given in combination (Figure 16). These data were consistent with previous in vivo studies examining the efficacy of panobinostat and bortezomib in PDX xenografts [123]. Further, carfilzomib alone had no beneficial effect on spontaneous lung metastasis but did not subtract from the efficacy of panobinostat when used in combination. These
data underscore the need for *in vivo* testing of potentially synergistic therapies identified *in vitro* but again support the use of HDAC inhibitors for the treatment of primary and metastatic osteosarcoma.

![Graphs and images](image)

**Figure 15-1:** Carfilzomib has no effect on primary osteosarcoma growth or lung metastasis *in vivo*.

a, b. K7M2, and SAOS2-LM7 cell lines were incubated with the indicated concentrations of panobinostat (PANO), carfilzomib (CARF) or panobinostat plus carfilzomib (CARF+PANO) for 48 hr. Cell viability (CellTiter blue) is presented as a percentage of vehicle control. c. Spider plot of K7M2 bioluminescence (RLU) over time in individual vehicle control (VEH; n = 6) carfilzomib (CARF; n = 7), panobinostat (PANO; n=6) or carfilzomib and panobinostat (CARF+PANO; n = 5) treated mice. Treatment was initiated 3 days subsequent to inoculation (blue arrow). Mice were removed from study upon reaching 1x10⁶ relative light units (RLU). Representative images show bioluminescence in each group at day 34 with hotter colors indicating greater tumor burden.
Figure 16-2: Carfilzomib has no effect on primary osteosarcoma growth or lung metastasis in vivo.

d. Average of bioluminescence in vehicle control and treated mice.  
e. Analysis of bioluminescence between the groups at day 34.  
f. Spider plot of K7M2 lung bioluminescence (RLU) over time in individual mice from each group.  
Representative images show bioluminescence in each group at day 34.
bioluminescence was observed by blocking tibia signal with light proof material. **g.** Analysis of average bioluminescence in spontaneous metastases arising from orthotopic primary K7M2 tumors. **h.** Kaplan Meier curve of time to progression (TTP) of lung bioluminescence detection in mice bearing primary (tibial) K7M2 tumors in each group. Asterisks denotes statistical significance (*p<0.05).

**Discussion:**

Although rare, osteosarcoma remains a deadly tumor type, especially for patients with metastatic disease. Despite therapeutic advances for several solid malignancies, the treatment paradigm for osteosarcoma has remained frustratingly static for 3 decades. Specifically, the 5-year event-free survival rate for osteosarcoma with no metastasis is 60%, but this drops to 28% if there is evidence of synchronous metastases [113]. Further, a retrospective analysis of 247 cases of osteosarcoma showed that 13% of patients present with synchronous lung metastases, while a further 21% will have metachronous lung involvement within 10 months [52], and these are responsible for the majority of osteosarcoma related deaths.

Clinical trials of Food and Drug Administration (FDA) therapies for osteosarcoma treatment are difficult and time consuming to conduct given the relative low incidence of disease. To identify effective therapies that will motivate clinical trials, we previously assessed the response of 5 osteosarcoma cell lines 143B, MNNG/HOS, MG63, U2OS, and SAOS2 to a panel of approved therapeutics [115]. We consistently observed that the HDAC inhibitor panobinostat compromised the survival of all osteosarcoma cell lines at low concentrations. These observations are consistent with studies examining the *in vitro* effects of panobinostat and other broad spectrum HDAC inhibitors such as vorinostat on osteosarcoma cell lines [109, 125]. Independent studies conducting an *in vitro* screen of drug efficacy on 3 osteosarcoma patient-derived xenografts (PDXs) also
identified HDAC inhibitors and specifically panobinostat as effective at inducing osteosarcoma cell death [124]. Importantly, panobinostat was noted to have additive cytotoxicity in vitro with chemotherapies commonly used to treat osteosarcoma, doxorubicin and gemcitabine [124]. Collectively, these in vitro studies underscore the potential for HDAC inhibition for the treatment of osteosarcoma.

To date, no studies have assessed the efficacy of panobinostat as a single agent on osteosarcoma in vivo. Here, using several complementary models of human and murine osteosarcoma, we show that panobinostat treatment effectively limits the growth of primary disease and spontaneous metastasis to the lung, and that it can also prevent seeding of the lung tissues by osteosarcoma cells. These data (Figure 7) suggest that applying panobinostat in a neoadjuvant or adjuvant setting for the treatment of primary disease could greatly reduce the incidence of, or time to, metachronous primary or metastatic relapse. Importantly, we also showed that panobinostat significantly reduced the growth of established osteosarcoma lung metastases and greatly extended overall survival. Panobinostat does have reported toxicities such as anemia and diarrhea at the doses and frequency used but mice quickly rebounded during the drug “holiday.” Given the significance of our results it is likely that panobinostat treatment at lower doses or over longer intervals would remain efficacious. Collectively, these data provide strong rationale for the design of clinical trials to test panobinostat in canine osteosarcoma and ultimately humans. We anticipate that patients diagnosed with lung metastatic osteosarcoma, who are at most risk and have failed other treatments, represents the focus cohort for such trials.
While presumably the effects of panobinostat are cancer cell intrinsic, there are also potential benefits on the tumor microenvironment. For example, panobinostat has been shown to prevent osteoclast formation and function [126]. Additionally, in other malignancies, HDAC inhibition in combination with HER2 blocking antibodies has been shown to activate immune cells such as natural killer (NK) cells to improve the tumoricidal effects on HER2 positive breast tumors [127]. In melanoma, panobinostat has been shown to not only inhibit the disease but to also augment the expression of major histocompatibility complex and co-stimulatory molecules in tumor cells, leading to the activation of antigen specific T-cells [128]. In support of this, the effects of panobinostat and romidepsin in our study were more striking in the syngeneic K7M2 immunocompetent model compared to the SAOS-LM7 immunocompromised model. We will be exploring this further as we develop our studies.

In our siRNA studies guided by the expression of HDACs, we found that HDAC1 and HDAC2 functionally contribute to the maintenance of osteosarcoma. Interestingly, silencing of HDAC6 promoted tumor growth but this effect was not observed with the HDAC6 inhibitor tubistatin, suggesting potentially non-catalytic tumor suppressive roles. Notably, analysis of public datasets reveals high expression of HDAC1 in PDX models of osteosarcoma compared to HDAC2 and other HDAC members [129]. Further, consistent with their roles in osteosarcoma cell fate, HDAC1 and HDAC2 are expressed in more mesenchymal/osteoblast precursor states, and their inhibition or deletion promotes osteoblastic differentiation [130]. Finally, although HDAC2 depletion has been reported to promote the stemness of MG63 osteosarcoma cells, and in vivo data
indicates a tumor suppressive role for HDAC2 [131], these studies did not assess if there are compensatory effects on HDAC1 expression.

The key HDAC1 and HDAC2 regulated targets that contribute to the maintenance of osteosarcoma biology remain to be elucidated but their identification could lead to the development of more specific targeted therapies for osteosarcoma treatment. Regardless, our studies provide a strong rationale for clinical trials that assess the efficacy of panobinostat or romidepsin for the treatment of metastatic osteosarcoma. To date, no trials with HDAC inhibitors have been conducted for osteosarcoma, and we submit these trials should be a high priority, particularly given the dearth of effective therapies for metastatic metachronous osteosarcoma, and the fact that both panobinostat and romidepsin are FDA-approved for the treatment of other malignancies

**Materials and Methods:**

**Cell lines:**

K7M2 (RRID: CVCL_V455) (a kind gift from Dr. Chand Khanna, NCI [118]), SAOS2 (RRID:CVCL_0548) and SAOS2-LM7(RRID:CVCL_0515) (a kind gift from Dr. Eugenie Kleinerman, MD Anderson, [119]), and primary murine mesenchymal stromal cells (MSCs) were isolated from the cortical bone of C57BL/6 mice. All cell lines were mycoplasma free (Cat # CUL001B, R&D Systems). All human cell lines were validated by short tandem repeat (STR Moffitt genomics core) and mouse cell lines via IDEXX BioAnalytics within the last 6 months. Cell lines were passaged in recommended culture medium supplemented with 10% fetal calf serum. All cell lines were transduced
to express luciferase using Cignal Lenti Positive Control (luc) (Qiagen CAT# 336891). Positive clones were selected using puromycin.

**HDAC qPCR, silencing and immunoblotting:**

Total RNA was extracted from cells with TRIzol (Invitrogen) per manufacturer protocols. HDAC human and mouse primers were synthesized (IDT) (Supplemental Table 1). RT-PCR mixtures were generated using SYBR Green reagent (Applied Biosystems, Cat#: 4309155) and reactions were performed and quantified using ABI-7900HT instrument and SDS 2.3 software per manufacturer protocols (Applied Biosystems).

Total RNA was extracted from cells with TRIzol (Invitrogen) as per manufacturer's instructions. HDAC human and mouse primers were synthesized (IDT) (Table 2). RT-PCR mixtures were generated using SYBR Green reagent (Applied Biosystems, Cat#: 4309155) and reactions were performed and quantified using ABI-7900HT instrument and SDS 2.3 software per manufacturer protocols (Applied Biosystems).

**Table 2: Human and mouse HDAC primers for qRT-PCR analysis**

<table>
<thead>
<tr>
<th>TARGET</th>
<th>HUMAN</th>
<th>MOUSE</th>
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<tbody>
<tr>
<td><strong>HDAC 1</strong></td>
<td>5'-GGTCGAAATGACCGATTCTCT-3’ 3'-TCGGAGAAACTCTCTCTCACAGG-5’</td>
<td>5'-TGAAGCCTCACCGAAATCCGCAAT-3’ 3'-TGGTCATCTCTCTCGCATTGC-5’</td>
</tr>
<tr>
<td><strong>HDAC 2</strong></td>
<td>5'-CTCAATGCACCTCGTTAGCCAGAT-3’ 3'-GCTATGGGTTGTCTGTGCTC-5’</td>
<td>5'-GTTTGTGACCTCTCTCGACCGGT-3’ 3'-CTGGGCATAGTGATGTCCCTCCAG-5’</td>
</tr>
<tr>
<td><strong>HDAC 3</strong></td>
<td>5'-AAGTTCTCTGATATCGGTTAACAG-3’ 3'-CGTATAACATAGCGAGCCAGAG-5’</td>
<td>5'-AACCTCATGCGCTGGCATGAC-3’ 3'-GTAGCCCTCGAATGGAGGCAGG-5’</td>
</tr>
<tr>
<td><strong>HDAC 4</strong></td>
<td>5'-CGGTGAGAGTTGGACTTACGGC-3’ 3'-GTGTAGAGCTGAAACTGTGG-5’</td>
<td>5'-ACCACAGTTCTGGAGAAGCA-3’ 3'-TCCGTGACTCCTCTCTTGCT-5’</td>
</tr>
<tr>
<td><strong>HDAC 5</strong></td>
<td>5'-GCCTCAATCTGACCCATACCC-3’ 3'-GGTGCTCTTTGCGTACAGAC-5’</td>
<td>5'-TCGCTGTCTCATCTACCTGCT-3’ 3'-GTCAAAGTGGCCACCTTCACCG-5’</td>
</tr>
<tr>
<td><strong>HDAC 6</strong></td>
<td>5'-TACAGCCAAGAAGGATGAGG-3’</td>
<td>5'-GTCAGCCAAGAAGGATGAGG-3’</td>
</tr>
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</table>
Table 2: (Continued)

<table>
<thead>
<tr>
<th>HDAC 11</th>
<th>3'-ACCACTCCTCAGCTCTGGAAAC-5'</th>
<th>3'-ACACTTCCCGTCATCAGGCCAC-5'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>5'-CTTCTGTGCCTATGCGGACATC-3'</td>
<td>3'-GAAGTCTCGCTCATGCCCATTG-5'</td>
</tr>
</tbody>
</table>

For analysis of protein levels by immunoblotting, cells were lysed with RIPA (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris, pH 8). Protein concentration was determined by BCA (Pierce, Waltham, MA, USA; #23225). Blots were blocked in 5% BSA for 1 hour followed by primary antibody. Primary antibodies for HDAC1, HDAC2, HDAC3, and HDAC5 were purchased from Cell Signaling (Cat # 2062, 57156, 85057 and 20458). HDAC6 antibody was purchased from Abclonal (Cat # A11259). HDAC8 and HDAC11 antibodies were a kind gift from Dr. Ed Seto (George Washington University). All primary antibodies were diluted 1:1,000 in blocking solution + 0.1% Tween-20, and incubated overnight at 4°C. Actin (Santa Cruz sc-1615 or Cell Signaling #3700) was used as a loading control. Blots were washed, then incubated with HRP-conjugated anti-species secondary (Cell Signaling Technology, Rabbit #7074/Mouse #7076, 1:1,000) and developed by enhanced chemiluminescence followed by exposure to light-sensitive film or imaging by LI-COR Odyssey Fc. Quantification performed with Image Studio software (LI-COR).

For HDAC1, HDAC2, and HDAC5 silencing, siRNA was purchased from Santa Cruz (Cat # sc-29344, sc29346, sc-35542, control siRNA sc-37007). HDAC6 siRNA was purchased from Dharmaco (Cat # L-043456-02-005). siRNA transfections were performed using Lipofectamine RNAiMAX Cat # 13778030) according to manufacturer protocols.
Cytotoxicity assays:

SAOS2, SAOS2 LM7 and K7M2 cells were seeded 5X10^3 cells per well in 96-well plates in triplicate and were treated with a dose range of panobinostat (Selleckchem Cat #S1030) (1.8, 2.4, 3.3, 4.5, 6.0, 8.1, 11.0, 14.8 and 20nM) or a dose range of romidepsin (Selleckchem Cat #S3020) (5.9, 8.8 13.2, 19.8, 29.6, 44.4, 66.7, 100 and 150nM). For dual treatment with Panobinostat and Carfilzomib (Selleck Cat #S2853) K7M2 and SAOS2 cells were plated at 5X10^3 cells per well in 96-well plates and treated with panobinostat at a dose range (1.4, 1.8, 2.5, 3.3, 4.5, 6.1, 8.2, 11.1 and 15nM) or carfilzomib at a dose range (1.9, 2.6, 3.5, 4.7, 6.3, 8.5, 11.5, 15.6 and 21nM) or combination of both drugs. IC_{50} values were determined at 48 hours post treatment using Cell Titer Blue assay (Promega, Cat # G8080) To determine the tubastatin (Selleckchem Cat # S2627) IC_{50} value K7M2 cells were seeded at 2X10^4 per well in 96-well plates and treated with the following concentrations (0, 1, 5, 10, 15, 100, 250, 500, 1,250, 2,500, 5,000,and 10,000 nM). All treatments were done in triplicate and cell growth was assayed at 48 hours using MTS Cell Titer 96-well proliferation assay (Promega Cat # G5421). The impact of panobinostat and romidepsin on PDX derived cell lines was examined in a publicly available dataset (https://braid.stjude.org/masttour/) [123].

In vivo studies:

All in vivo studies were performed under the Moffitt/University of South Florida Institute of Animal Care and Use Committee approved protocol R3540 (CCL). For in vivo experiments K7M2 or SAOS2-LM7 luciferase-expressing cells were injected into BalB/c
or NSG mice, respectively. For the primary osteosarcoma study 1X10^5 cells were injected intratibially in 20μl of sterile PBS into one of the hind tibias. The other hind tibia was injected with 20μl of sterile PBS as a control. For the lung seeding studies 1X10^6 K7M2 cells were injected intravenously via tail vein. For treatment with panobinostat (10mg/kg in 10mM Citrate buffer with 10% Captisol, (Cydex pharmaceuticals, Cat # Rc-OC7-100) mice were randomized into cohorts for treatment by intraperitoneal injections in 200-μl volumes of either panobinostat or vehicle for the remainder of the study (5 days on, 2 days off treatment). Treatments with romidepsin 2.4mg/kg dissolved in 2%DMSO, 30% 400PEG, 5% Tween 80 in ddH20 were administered twice a week subcutaneously in 100-μl volumes. Treatment with Carfilzomib (2mg/kg in 10mM Citrate buffer with 10% Captisol) was administered by tail vein injection in 100-μl volumes on 2 consecutive days followed by 5 days no treatment. In the dual panobinostat carfilzomib treated mice group we treated with 1/10 of the dose of the individual drugs (0.2mg/kg carfilzomib and 1mg/kg panobinostat). Each week, mice received both carfilzomib and panobinostat on days 1 and 2 followed by three days of panobinostat only and then two days no treatment. Bioluminescence for all studies was measured as a correlate of tumor growth (IVIS™, Perkin Elmer).

**Ex vivo bone analysis:**

Tibias were collected and fixed in 10% formalin for 24-48 hours then transferred to 50% ethanol. Radiographic images (Faxitron, X-ray Corp) were obtained using energy of 35kVp and an exposure time of 8 milliseconds. The spatial resolution is 10 lp/mm (48μm). The tumor volume (TuV) was calculated as a function of the total tissue volume (TV) of the tibial medullary canal using ImageJ software. For μCT analysis, the proximal
tibia metaphyses were scanned (μCT-40; Scanco Medical). An evaluation of trabecular bone structural parameters was performed in a region that consisted of 1mm starting at 500µm from the growth plate. A three-dimensional cubical voxel model of bone was built, and calculations were made for relative bone volume per total volume and trabecular number. After X-ray and μCT analysis, tibias were decalcified (14% EDTA, pH 7.4, 3 weeks), processed, and paraffin embedded.

**Immunofluorescence:**  
For paraffin embedded tissues, slides were dewaxed and rehydrated to water. Antigen retrieval was performed with heat 5 minutes in a pressure cooker (1X Tris EDTA pH 9). Slides were blocked in 10% goat serum in 1X TBS for 1 hour at room temperature. Primary antibodies Phospho-Histone H3, Cell Signaling Cat #9701L, 1:400 dilution; Cleaved Caspase 3 1:200 dilution, Cell Signaling Cat #9661S, were diluted in 10% normal goat serum (Vector Laboratories Cat # S-1000) and incubated overnight at 4°C in a humidified chamber. After 3 washes in 1X TBST followed by 1 wash in 1X TBS, secondary antibodies (Alexa Fluor™ Goat Anti Rabbit 568, (Thermo Fisher Scientific #A-11011); Alexa Fluor Goat Anti Mouse 488, (Thermo Fisher Scientific #A32723) were incubated at a 1:1,000 dilution in 10% normal goat serum for 1 hour at room temperature. Slides were washed three times in 1X TBS and mounted using Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, # H-1200). Slides stored under light-proof conditions until image acquisition.

For in vitro immunofluorescent analyses, cells were seeded into 8-well chamber slides (LAB-TEK #154534) at 2x10^4 and cultured overnight before treatment with either
50% MSC CM or DMEM 5% FBS or 100nM etoposide for 5 hours. Cells were then rinsed with PBS and fixed in 4% PFA at room temperature for 20 minutes. Fixed cells were then blocked for 30 minutes at room temperature in antibody diluting buffer (2% BSA, 0.1% Triton X-100 in PBS). Primary antibodies (Acetyl-Histone H3, Millipore #06-599, 1:400 dilution in antibody diluting buffer; Rabbit IgG Isotype Control, Thermo Scientific #31235) were incubated at room temperature for 30 minutes. Cells were then washed 3x in PBS and incubated with secondary antibody (Alexa Fluor™ Goat Anti-Rabbit 488, Invitrogen #A-11034, 1:1000 dilution in antibody diluting buffer) for 30 minutes at room temp in the dark. After washing 3x in PBS, culture chambers were removed, and the slides were mounted with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, # H-1200). Mounted slides were stored under light-proof conditions at 4°C until microscopic analysis. For quantitation of immunofluorescent stained samples, 3 representative images were acquired at 40X magnification, and the ratio of positive stained cells to total number of cells per field (using DAPI) were calculated using ImageJ.

**Statistical analysis:**
To determine statistical significance among groups, T-test or analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was performed. A p-value <0.05 was considered as statistically significant. Data are presented as standard error from the mean (S.E.M). All statistical analyses were performed with Graph Pad Prism 6.0 (GraphPad Inc. La Jolla, CA).
Chapter 4: Metastatic Skeletal Malignancy and Prostate Cancer:

Introduction:
Metastasis marks a turning point for many cancers, evolving from a disease that can generally be successfully treated to one for which cure is unlikely with present therapies. This is due in part to the indispensable nature of the organs involved. The skeleton is one of the most common sites of metastasis, with prostate and breast cancer comprising nearly 70% of all cases [132-135]. Lung, kidney, thyroid, bladder, melanoma, and renal cell carcinoma can also metastasize to the bone. Bone metastases are currently incurable and extremely painful. Most currently available therapies are palliative and fail to increase overall survival.

The bone microenvironment is comprised of many different cell types including hematopoietic cells, stromal cells (e.g. fibroblasts and mesenchymal stem cells), endothelial cells, adipocytes, chondrocytes, osteoblasts, and osteoclasts [136]. Understanding the roles and interaction of these cell types in the bone microenvironment and tumor cells can provide key insights into new therapeutic options for patients with bone metastases.

Prostate Cancer Overview:
The prostate is an organ that is part of the male reproductive system. Normally the size of a golf ball, its main function is to secrete fluid that protects the sperm during ejaculation. As men age; the prostate commonly grows larger in size, a condition
diagnosed as benign prostatic hyperplasia (BPH) [137]. Although BPH is not cancer, it can result in some of the same symptoms such as frequent need to urinate and difficulty urinating. Prostatic intraepithelial neoplasia (PIN), is a precancerous condition defined by the presence of abnormal prostate cells that can be categorized as either low or high grade. Men with high grade PIN are more likely to develop prostate cancer [138, 139]. Initially, prostate cancer is localized to the prostate gland, but as it progresses to advanced prostate adenocarcinoma, there is often local invasion. If left untreated, the cancer can become metastatic and spread to distant sites, commonly the lymph nodes and skeleton.

Prostate cancer stage is defined by histological analysis of biopsies using a numeric grading scale defined by Donald Gleason in the 1970s and refined several times since [140] [141, 142]. The score is calculated by assigning a value between 1-5 based on the abnormality of the tissue with 5 being the most abnormal. There are two scores given by analyzing the 2 biggest tumor sections found in the microscopic analysis of a biopsy. The two scores are added together for a final score. Gleason score prognosis is defined using the International Society of Urological Pathology (ISUP) grading system shown in (Table 3) [143].

One of the defining features of prostate cancer are hormones. Specifically, androgens such as testosterone drive the growth of this cancer. The discovery of hormone ablation as a treatment for prostate cancer lead to a Nobel prize for Charles Brenton Huggins [144]. Castration or androgen deprivation therapy is a valuable therapy for advanced prostate cancer patients still used today, however in almost all cases, the
cancer eventually progresses in the absence of androgen and becomes known as castrate resistant prostate cancer (CRPC) [145].

Table 3: Table of (ISUP) grading system and prognostic risk in relation to Gleason Score [143]

<table>
<thead>
<tr>
<th>Gleason Score</th>
<th>ISUP Grade</th>
<th>Prognostic Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 6</td>
<td>Grade 1</td>
<td>Low Risk</td>
</tr>
<tr>
<td>7 (3+4)</td>
<td>Grade 2</td>
<td>Intermediate (Favorable)</td>
</tr>
<tr>
<td>7 (4+3)</td>
<td>Grade 3</td>
<td>Intermediate (Unfavorable)</td>
</tr>
<tr>
<td>8</td>
<td>Grade 4</td>
<td>High</td>
</tr>
<tr>
<td>9-10</td>
<td>Grade 5</td>
<td>High</td>
</tr>
</tbody>
</table>

Prostate Cancer Statistics:
Prostate cancer is the 2nd most diagnosed cancer in men with approximately 165,000 new cases diagnosed and almost 30,000 deaths estimated in 2020. To put these numbers into a different perspective, 1 in 9 men will be diagnosed with prostate cancer in his lifetime, and 1 in 41 will die from prostate cancer [146]. If prostate cancer is
diagnosed before metastasis, the 5-year survival rate is approximately 99% but once the cancer spreads to a distant site, the survival rate decreases to 30% [147]. The most common site for prostate cancer metastasis is the skeleton, but other sites can include lymph nodes, lungs, liver, brain, and less commonly to adrenal glands, breasts, eyes, kidneys, muscles, pancreas, salivary glands, and spleen [146]. In a study that analyzed 1,589 autopsies of men with advanced prostate cancer, 90% had evidence of bone metastases [148]. Since bone is the most common site of prostate metastasis, our studies focus on understanding the skeleton as a metastatic site for prostate cancer.

**Prostate Cancer Risk Factors:**

Some common risk factors for prostate cancer include age, race, genetics, exposure to radiation, smoking, and obesity [149]. The average age of prostate cancer at 66 has decreased since prostate specific antigen (PSA) screening for prostate cancer became common. PSA is a serine protease that is produced by prostate epithelial cells and prostate cancer cells. Rising serum PSA levels in serum can indicate prostate cancer progression, but there is controversy regarding the reliability of PSA and improved biomarkers are urgently needed [150, 151]. Age is a primary risk factor for prostate cancer, with only 10% men diagnosed in 2012 under the age of 55. [152, 153]. African American men are more than 60% more likely to develop prostate cancer compared to Caucasian men. [154]. Having a family history of prostate cancer is a risk factor [155, 156]. Environmental and behavioral factors are also implicated. Chemical and radiation exposure increase the risk of developing many types of cancer, and radiation therapy for prostate cancer in some cases is the cause of second primary cancer formation [157, 158]. Smoking is generally associated with increased risk for lung cancer but
increases the risk of many cancer types including prostate cancer [159]. Obesity is a risk factor for many diseases including prostate cancer. For example, greater body mass index (BMI) is correlated with a significant higher risk of prostate cancer mortality [160]. Conflicting literature suggests it is possible that increased height may also increases the risk of developing prostate cancer [161, 162].

**Prostate Cancer Genomics:**

The most notable genomic abnormality in prostate cancer is the gene fusion of transmembrane protease serine 2 (TMPRSS2) and members of the E26 transformation-specific (ETS) transcription factors [163]. The ETS transcription factors are a group of 29 different genes divided into 12 subgroups that share common DNA binding domains or ETS domains [164, 165]. These transcription factors are important in regulating many cellular processes including apoptosis, cell proliferation, differentiation, angiogenesis and metastasis [166]. The most common ETS gene found overexpressed in nearly 50% of prostate cancers is the oncogene ETS-related gene (ERG) [167]. The fusion with androgen regulated TMPRSS2 is frequently responsible for the overexpression of ERG [163]. The TMPRSS2/ERG fusion commonly leads to overexpression of the oncogene c-MYC (MYC) [168]. Genetic alterations in the chromosomal location of the androgen receptor (AR), Xq11-12 are also common in prostate cancer patients. Sequencing of 50 samples obtained from treated CRPC patients after rapid autopsy confirmed a list of genes that have been reported to be mutated in prostate cancer. The genes included TP53, PTEN, APC, ZFHX3, and RB1 [169].
Current Therapies for Prostate Cancer:

Treatment for prostate cancer is dependent on the disease stage, age, and health of the patient. Early stage prostate cancer that is localized and has a Gleason score of 6 or below is often monitored with active surveillance or watchful waiting. It often can be years before progression of tumor growth requires any treatment, and side effects of treatments include erectile dysfunction and incontinence which negatively affect quality of life. Since most men are elderly when diagnosed with prostate cancer, many men do not undergo treatment and instead elect for active surveillance [170-172]. When a patient’s Gleason score indicates high-risk prostate cancer, the recommended therapy is typically surgery (radical prostatectomy) followed by radiation therapy and ADT; chemotherapies are sometimes co-administered with ADT commonly if other therapies fail [146, 173]. Radiation therapy options for prostate cancer include external beam radiation (EBRT) and brachytherapy. EBRT when the prostate is targeted with beams of radiation. There are multiple different options for EBRT they include three-dimensional conformal radiation therapy (3D-CRT), intensity modulated radiation therapy (IMRT), stereotactic body radiation therapy (SBRT), and proton beam radiation therapy [174]. Brachytherapy or internal radiation therapy is when small radioactive pellets are inserted directly into the prostate. In high-risk cases, brachytherapy can be combined with EBRT [175].

ADT or hormone therapy is used to block the male sex hormone testosterone from activating AR signaling that fuels prostate cancer cell growth. AR is a nuclear hormone receptor, which serves as a transcription factor that regulates gene expression responsible for the formation of the male reproductive system, including the prostate
AR is activated and translocated to the nucleus by the binding of its ligands androgens. Androgens are androstenedione, testosterone and dihydrotestosterone (DHT) [177]. Androstenedione is a testosterone precursor, and testosterone is converted to the biologically active DHT by 5α reductase which has a 2-fold higher binding affinity to AR [178]. Testosterone production begins when chemicals in the brain stimulate testosterone production in the testes. When the body detects low testosterone levels, the hypothalamus begins producing luteinizing hormone-releasing hormone (LHRH) that stimulates luteinizing hormone (LH) production by the pituitary gland. LH then stimulates production of testosterone by the testes [179]. The most radical form of ADT is orchiectomy or surgical removal of the testes this is irreversible but is also cost effective compared to other treatments [180]. There are pharmaceutical approaches that can be administer in lieu of the surgical route, including chemical castration or antiandrogen therapy. There are LHRH agonists and antagonists that both block the production of testosterone, these drugs include leuprolide, goserelin, triptorelin, histrelin, and degarelix. Anti-androgen drugs such as bicalutamide, nilutamide and flutamide block the testosterone function by binding and blocking AR. These drugs are often administered in combination with LHRH agonists [181].

Unfortunately, ADT therapy generally fails within 1-3 years, at which point the disease progresses to a hormone refractory state known as CRPC. There are multiple mechanisms by which PCa becomes castrate resistant. Many of these involve changes to the androgen receptor, including amplification, mutation, or expression of splice variants leading to constitutive activation [182] [183, 184]. AR amplification is observed in approximately 80% of patients [185]. Mutations in the ligand-binding regions of AR
causes AR antagonists, such as flutamide, bicalutamide and enzalutamide that typically work by binding AR and blocking the binding of androgens, to act as agonists activating AR and promoting prostate cancer progression [186-189]. Other signaling pathways can also become hyperactive following androgen deprivation therapy. In approximately 40% of primary prostate cancer, PI3K/AKT signaling pathway is altered, whereas it is altered in almost 100% of metastatic CRPC [190]. Amplification of c-Myc also occurs in approximately 70% of patients after ADT [191]. Consequently, metastatic disease must be targeted differently from the original primary cancer.

The treatment strategies for CRPC often includes chemotherapy. According to the National Comprehensive Cancer Network (NCCN) the most common chemotherapy used for advanced prostate cancer is docetaxel. Patients that do not respond to docetaxel can be given cabazitaxel, but at the risk of severe side effects. Mitoxantrone hydrochloride, is another chemotherapeutic option [146, 192]. Second generation ADT therapies are also available to CRPC patients. Abiraterone is an anti-androgen drug that can be effective in treating CRPC by inhibiting the cytochrome p450 enzyme 17R-hydroxylase17,20-lyase (CYP17A1), an enzyme needed for the production of androgens. In 2011 abiraterone was FDA approved for the treatment of CRPC [193, 194]. Another anti-androgen drug FDA approved in 2012 for CRPC patients is enzalutamide. Enzalutamide can block AR from translocation into the nucleus as well as prevent DNA binding of AR [195]. Another option for CRPC patients is a dendritic cell vaccine, sipuleucel-T. Currently sipuleucel-T is the only FDA approved immunotherapy available for prostate cancer receiving approval in 2010 [196].
Therapies for bone metastatic prostate cancer are mainly palliative with the exception of radium 223. Radium 223 is a calcium mimetic with the ability to bind hydroxyapatite, and therefore, targets areas of the skeleton where bone is actively being remodeled. It simultaneously emits alpha particles that cause irreversible double strand DNA breaks [197]. The FDA-approved radium 223 for bone metastatic CRPC in 2013 after it was shown to increase median overall survival form 11.2 months to 14 months [198, 199]. Most therapies for bone metastatic patients aim to prevent bone destruction by targeting osteoclasts. These drugs include bisphosphonates and denosumab. Bisphosphonates, as the name suggests are a group of chemical compounds consisting of two phosphate groups, they are derived from modifications to inorganic pyrophosphate (PPi). In the 1960s PPi was found to bind hydroxyapatite crystals and inhibit calcification [200]. Bisphosphonates are recruited to areas of active bone remodeling, where they are taken up by bone-resorbing osteoclasts, leading to their apoptosis [201]. There is also evidence that bisphosphonates can protect against pathological bone fracture by promoting survival of osteoblasts and osteocytes [202].

Receptor activator of nuclear factor-κB ligand (RANKL) is produced by osteoblasts and binds the RANK receptor on osteoclast precursors and promotes the formation of mature bone resorbing osteoclasts. Denosumab is a monoclonal antibody that binds RANKL and inhibits the binding to RANK on osteoclast precursors. This reduces osteoclast formation and activity [203, 204]. Denosumab does not increase overall survival of bone metastatic prostate cancers but does reduce skeletal related events (SRE) reduces bone pain and increases quality of life. The current treatments for bone metastatic prostate cancer are limited and mainly palliative with the exception of a slight
survival increase with the use of Radium 223. Understanding and further exploiting the interactions of the cancer cells and the bone microenvironment may lead to new therapeutics that are desperately needed.

**Vicious Cycle of Bone Metastasis:**

The vicious cycle is a paradigm that describes the symbiotic relationships between the bone microenvironment and the tumor cells that metastasize to the bone. A simplistic explanation of the vicious cycle includes three main cell types: osteoblasts, osteoclasts, and tumor cells. Osteoblasts are the cells that produce new bone, when mature they secrete extracellular matrix consisting of alkaline phosphatase, osteocalcin and type 1 collagen; this matrix is also known as the osteoid. The osteoid is then mineralized into hardened bone by hydroxyapatite [205]. Osteoclasts are the cells that degrade bone. Mature multinucleated osteoclasts from a seal with their “ruffled membrane” with the bone surface they then secrete hydrochloric acid and proteases most notable Cathepsin K, which demineralizes the bone. The osteoclast endocytoses the demineralized bone and factors trapped in the bone, and it is secreted though its apical surface [206, 207]. The third cell type is the tumor cell. When the tumor cells establish in the bone microenvironment, they secrete growth factors and cytokines such as VEGF, PDGF, ET-1, BMPs, PTHrP, various interleukins and other molecules. The osteoblast precursors MSCs are stimulated and differentiate into mature activated osteoblasts [208]. These osteoblasts express RANKL and macrophage-colony stimulating factor (M-CSF). The RANKL binds RANK and M-CSF binds c-fms on osteoclast precursor cells, monocytes. Under this stimulation by osteoblasts the monocytes fuse and become multinucleated osteoclasts. As the osteoclasts degrade bone they release
many different growth factors that are trapped in the bone matrix such as, TGF-βB, IGFs, Ca+, and other factors that feedback and fuel the growth and survival of the tumor cells completing the cycle [209]. Those 3 cell types are commonly used to describe the vicious cycle but there are many other cells in the bone marrow microenvironment that are also involved. They include but not limited to hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) endothelial cells, macrophages and MDSCs [210]. Understanding how to interrupt this cycle could provide therapeutic options to treat and cure bone metastases.

HSCs reside in the bone marrow and are the cells that give rise to all of the different blood and immune cells [211]. The HSC niche is located on endosteal regions in close proximity to the locations where osteoblasts reside. The survival, quiescence and proliferation of HSCs is regulated by osteoblasts produced cytokines [212-214]. MSCs are the precursors to the bone forming osteoblasts and also secrete many different cytokines and chemokines into the bone microenvironment. For example, MSC-induced tumor derived lysyl oxidase (LOX) promotes breast cancer metastasis [215]. MSCs also secrete VEGF, promoting angiogenesis and metastasis [216].

The vasculature plays important roles in bone metastasis, including promoting tumor cell dormancy. Dormancy is a challenging clinical aspect of metastatic disease, as disseminated tumor cells can remain undetected in protective niches until they awaken years, sometimes decades, later to form macrometastases [217, 218] Endothelial cells produce thrombospondin-1 that promotes the dormancy of breast cancer cells [219]. Osteoblasts also contribute to dormancy in the bone by production of
growth arrest specific 6 (GAS6) that when binds with its receptor AXL on disseminated prostate cancer cells triggering TGFβ2 signaling. [220, 221].

It is becoming increasingly evident that immune cells are involved in the vicious cycle [210]. Macrophages present in the bone marrow can play important roles in tumor progression and fueling the vicious cycle. Macrophages can be both pro- and anti-tumor and are can be classified as either M1 or M2 phenotypes. Anti-tumor M1 macrophages create an inflammatory environment by producing cytokines such as interleukin 1 (IL-1) interleukin-9 (IL-9) and tumor necrosis factor (TNF). They also produce nitric oxide (NO) and reactive oxygen intermediates (NOI) that can cause tumor apoptosis [222]. M2 macrophages produce anti-inflammatory cytokines such as interleukin 10 (IL-10) CCL17 and CCL18. and have pro-tumor roles [223]. Tumor cells produce chemokines such as CCL2, M-CSF, and VEGF that recruits macrophages into the tumor microenvironment. These macrophages are referred to as tumor-associated macrophages (TAMs). Due to other factors produced by the tumors such as IL10, IL-4 and IL-13 TAMs become polarized to the M2 phenotype [224]. Macrophage precursors, monocytes that reside in the bone marrow can also become myeloid derived suppressor cells (MDSCs). MDSCs can suppress T-cell activation and promote tumor growth and metastasis. They also have direct roles in the vicious cycle due to their ability to differentiate into osteoclasts [225]. MDSCs produce large quantities of TGF-β, which induces PTHrP production by tumor cells, this reestablishes the vicious cycle with stimulation of osteoblast derived RANKL [226].

Halting the vicious cycle will require careful study of the factors and cell types involved in regulating disease progression. It is presently unclear why prostate-bone
metastases tend to be osteogenic, whereas other cancers such as breast produce osteolytic metastases. Factors produced by primary prostate cancer cells in the vicious cycle such as WNTs, bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs) insulin-like growth factors (IGFs) have profound effects on MSC and osteoblast biology and are likely to play a role in the formation of osteogenic lesions [227-230].

Mesenchymal Stem Cells:

MSCs are pluripotent cells that can reside in almost every tissue in the body, having been isolated from many different tissue types, including bone marrow, cortical bone, amniotic fluid, adipose tissue, fetal tissues, dental pulp, and umbilical cord. [231-236] [237]. The bone marrow is an especially rich reservoir for these cells, where they account for approximately 1 out of every 10,000 bone marrow cells [238]. MSCs have the ability to differentiate under appropriate stimulus toward several lineages, including chondrocytes (cartilage), adipocytes (fat) and osteoblasts (bone) [239]. There is also evidence that they can form non-mesenchymal tissue such as gut, lung, liver and skin epithelium [240-243]. There is still debate on whether the cells that can differentiate into non mesenchymal tissues are mesenchymal stem cells or if the origin of these tissues is actually another population of multipotent adult progenitor cells. [241] MSCs are recruited to wound sites, where they play an active role in repairing damaged tissue [244, 245]. There are many cellular markers for MSCs, however many are not exclusive and can differ depending on the source of the MSCs. Human MSCs do not express hematopoietic markers and are generally negative for CD45, CD34, and CD14. The following markers are generally accepted as markers that are expressed by MSCs: CD105, CD73, CD44, CD90, Stro-1 and CD29 [240, 246-248].
MSCs and Their Dynamic Roles in Cancer Progression:

The idea that cancer is a disease of chronic inflammation was proposed in 1858 by Rudolph Virchow [249]. Since that observation, cancer has been described as wounds that do not heal [250]. MSCs are attracted to sites of inflammation and consequently are also attracted to the sites of tumors [251, 252]. There is conflicting information as to whether MSCs promote or inhibit tumor growth, and this seems to depend on the stage of cancer progression and tissue context. [253, 254] MSCs are pro-tumorigenic in many different cancers including breast, osteosarcoma, melanoma, lung, prostate and colon [255-262], and this occurs by multiple mechanisms. It is well documented that MSCs can differentiate into carcinoma associated fibroblasts (CAFs) and secrete tumor promotive growth factors such as stromal derived factor-1 (SDF-1) [263-265]. MSCs can also suppress immune cells as part of their wound healing activities, contributing to the pro tumor effects of MSCs. For example, in an allogeneic model where B-16 melanoma cells were implanted with or without MSCs, tumors only formed in the presence of MSCs, demonstrating the immune suppressive pro-tumorigenic effects of MSCs [258]. MSCs suppress the immune system by secretion of factors such as prostaglandin E2 (PGE2), interleukin 6 (IL-6), IL-10, and indoleamine 2,3 deoxygenase (IDO) [266-270]. MSCs can also promote tumor vascularization by secretion of pro-angiogenic factors such as VEGF and FGF-2 [216]. In colorectal cancer, MSC-derived neuroregulin 1 (NRG1) activates HER2/3 and AKT signaling to promote cancer cell survival [271]. Further, MSCs can promote metastasis by secretion of factors such as PDGF and TGFβ which in turn activate EMT and promote transcription factors in tumor.
cells like Snail, Slug and TWIST [272, 273]. For example, when breast cancer cells are co-cultured with MSCs epithelial genes become downregulated and EMT associated genes are upregulated [274]. MSCs suppress apoptosis through the upregulation of B cell lymphoma 2 (BCL2), by secretion of VEGF and FGF-2 [275, 276]. Multiple chemokines and cytokines secreted by MSCs can be pro-tumorigenic. For example, MSCs recruited to the breast cancer microenvironment secrete chemokine ligand 5 (CCL5) that promotes cancer cell invasion and metastasis [255]. In hepatocellular carcinoma, MSC-derived interleukin 6 (IL-6) promotes the progression promotes cancer progression via activation of STAT3 [271, 277]. MSCs have also been shown to promote osteosarcoma by activation of STAT3 leading to drug resistance [257].

Many studies have reported anti-tumorigenic effects of MSCs in various cancer types as well, including some that have also shown pro-tumorigenic effects, such as Kaposi’s sarcoma, liver, breast, pancreatic, lung, melanoma, glioma, non-Hodgkin’s lymphoma and multiple myeloma [278-286]. In an in vivo model of Kaposi’s sarcoma, human MSCs injected intravenously inhibited tumor growth by downregulation of AKT signaling in the tumor cells [278]. Wnt-signaling has also been shown to be downregulated by MSCs. For example, breast cancer cell growth was inhibited by MSC-derived DKK-1 which downregulated Wnt signaling [280]. Human MSCs were also shown to inhibit the growth of liver cancer cell lines both in vitro and in vivo, increasing apoptosis and decreasing proliferation by downregulating anti apoptotic factors like Bcl-2 survivin, and c-Myc [279]. Adipose derived MSCs inhibited pancreatic cancer growth both in vitro and in vivo by inducing cell cycle arrest but not apoptosis [281]. MSCs express the pro-apoptotic factor fas ligand (FasL) that normally promotes
wound healing by promoting the apoptosis of T-cells [287]. MSC derived FasL has been shown to trigger multiple myeloma cell apoptosis both in vitro and in vivo [286].

The effects MSCs have on tumorigenesis can also be dynamic, where for example they impair progression during the initial stages of hepatocarcinoma but support later stages of the disease [288]. The opposing effects of MSCs on tumor progression may in part be explained by the tissue source. Specifically, MSCs can be derived from several sources – bone marrow, umbilical cord blood, peripheral blood, dental pulp and adipose tissue – and MSCs from each tissue source differ in their capacity to either promote or inhibit cancer growth [289].

**MSCs and Prostate Cancer:**

MSCs have been shown to promote the metastasis of primary prostate cancer. Prostate cancer cells express the cytokine chemokine (C-X-C motif) ligand 16 (CXCL16), which recruits MSCs that express C-X-C chemokine receptor type 6 (CXCR6) or CD168. The CXCL16/CXCR6 signaling cascade leads to the transformation of MSCs into cancer associated fibroblasts (CAFs) that express C-X-C motif chemokine 12 (CXCL12) also known as stromal cell-derived factor 1 (SDF-1). The CXCL12 expressed by CAFs binds to CXCR4 on tumor cells promoting EMT and metastasis. [263] This work provides insight into the role of MSCs in primary prostate cancer, however the roles of MSCs on bone metastatic prostate cancer are still not fully understood.
Chapter 5: Mesenchymal Stem Cell-Derived Interleukin-28 Can Drive the Selection of Apoptosis Resistant Bone Metastatic Prostate Cancer

Rationale:

Bone metastatic prostate cancer promotes mesenchymal stem cell recruitment and their differentiation into osteoblasts. However, the effects of bone-marrow derived MSCs on prostate cancer cells are less explored. Here, we explore the effects of those interactions. We and others have reasoned that defining how prostate cancer cells establish and grow in the bone microenvironment may reveal new therapeutic targets for treatment [135, 290, 291]. We aim to understand how MSCs promote the evolution of prostate cancer cells in bone microenvironment through secretion of IL-28 and activation of Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway in prostate cancer cells.

Interleukin 28 (IL-28) also known as interferon λ (IFN λ) is a cytokine that was discovered in 2003 [292, 293]. IL-28 is induced by viral infection and is secreted by both macrophages and dendritic cells to exert anti-viral activity [294]. IL-28 has 2 isoforms IL-28A and IL-28B that are 96% homologous. Both isoforms have a similar amino acid sequence to interleukin 29 (IL-29) [292]. Together, these 3 interleukins make up the type III interferon family and are clustered together on human chromosome 19 or murine chromosome 7 [295]. IL-28 signals through a heterodimer receptor made up of IL-28Rα and IL-10Rβ [296].
The JAK/STAT pathway is activated by IL-28 binding its receptor allowing JAK1 and TYK2 to bind the cytoplasmic ends of the receptor complex and activate latent STATs 1, 2, 3 and 5. [297] Since its discovery, IL-28 has been shown to have multiple different functions including anti-tumor activities [298-300]. Other cytokines and growth factors can activate the signaling pathway. Depending on the ligand binding, the receptors can form homodimers, heterodimers, or trimers. These receptor complexes can then become activated by JAKs that cross phosphorylate each other and phosphorylate of the C-terminus of the receptor complex. There are four different JAKs, JAK1, JAK2, JAK3 and Tyk2 that are selective for specific receptors. Originally JAK1 and JAK2 were discovered by a PCR screen from protein kinases and were called “just another kinase” but were renamed Janus kinase after the 2 faced Roman god because of the two phosphate transferring domains that play opposite roles in the kinase activity of the proteins [301].

STATs have SRC homology 2 (SH2) domains that can bind phosphorylated receptor complexes where they are phosphorylated and allow the production of either homodimers or heterodimers with other STAT members. There are STAT inhibitors that disrupt the SH2 domain and inhibit STATs from dimerizing and acting as transcription factors [302]. Seven different STATs that have been discovered, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [303]. Once phosphorylated and dimerized STATs translocate to the nucleus where they directly bind DNA and regulate gene expression. [304-308]. The JAK/STAT pathway has normal roles in regulation of inflammation and apoptosis, the dysregulation of the pathway can result in diseases of the immune system and cancer. STAT3 has been implicated in cancer progression and
metastasis and has been described as an oncogene [309, 310]. In one study, 95% of 223 prostate bone metastasis samples were positive for pSTAT3. We propose that using a small molecule STAT inhibitor, we can treat bone metastatic prostate cancer.

**RESULTS:**

**MSCs have dynamic effects on prostate cancer cell growth:**

Human [311] and mouse bone metastatic prostate cancer specimens were initially assessed for MSC content by immunohistochemistry (IHC). As predicted [312, 313], there were abundant numbers of α-SMA positive MSCs in both human and mouse bone metastatic prostate cancer (Fig. 13a). These findings were confirmed using additional markers for human (CD90) and mouse (nestin) MSCs (Figure 17) [313-315]. To assess the possible effects of MSCs on prostate cancer cells we isolated mouse bone MSCs (CD29+SCA1+CD45−) and confirmed their stemness properties by performing osteoblastic, adipogenic and chondrogenic differentiation assays (Figure 20). Using a modified Boyden chamber assay, we observed MSCs are highly chemotactic to prostate cancer cell line-derived conditioned media compared to serum-free control (Figure 19 b). To assess the potential effects of MSCs on prostate cancer cell fate, the rodent prostate cancer cell line PAIII [316, 317] was directly co-cultured with mouse MSCs. Notably, MSCs significantly inhibited prostate cancer cell growth even down to 1:10 ratios of MSC to PAIII cells (Figure 19 c). Further, a dose-dependent inhibition of prostate cancer cell growth was evident when they were cultured with MSC conditioned media (CM); thus, a soluble MSC-derived factor suppresses prostate cancer cell growth (Figure 19 d). A significant rounding of PCa cells combined with detachment was noted
within 6 hours of culture with CM, suggesting that the MSCs triggered PCa cell apoptosis. Consistent with this, high levels of cleaved caspase-3 are manifest in MSC CM-treated PCa cells compared to controls within 6 hours (Figure 19 e). This effect was not limited to PAIII PCa cells or to mouse MSC, as both mouse and human MSC conditioned medium impaired the growth of mouse Myc-CAP and human RWPE and DU145 prostate cancer cells (Figure 19 f). By contrast, culture in MSC CM increased the growth rates of V-CAP, 22RV1, LNCaP and the LNCaP derived C4-2B prostate cancer cell lines compared to controls, underscoring the complexity of MSC effects on prostate cancer cell behavior (Figure 19 f). Of note, the negative effects on prostate cancer cell growth were MSC-specific, as conditioned medium derived from osteoblasts failed to affect PCa cell growth (Figure 19 g). Finally, MSC CM also did not affect the growth of normal prostate epithelial cells (PREC; Figure 19 h).

Prostate cancer conditioned media promotes osteogenesis of MSCs:

Prostate cancer has a unique phenotype when it metastasizes to the bone. Whereas most cancers that metastasize to the bone, such as breast and lung cancers, produce osteolytic lesions and loss of bone mass, prostate cancer typically produces mixed lesions with both osteosclerotic and osteolytic lesions. To determine if secreted factors from prostate cancer cells is responsible for the increased bone formation commonly observed in prostate to bone metastases MSC were differentiated in the presence or absence of conditioned media from prostate cancer cells. Conditioned media from prostate cancer cell lines was used in combination of osteogenic supplements to assess the effect of osteogenic differentiation.
Figure 17: MSC infiltration in bone metastatic prostate cancer.

Representative images of CD90 staining (red; arrows) in human samples of bone metastatic prostate (n=10; three representative patients are shown) or nestin staining (red; arrows) in tissue sections derived from mouse tibias bearing PAIII bone metastases (three representative mice are shown). For human and mouse specimens, pan-cytokeratin (pCK; green) was used to localize prostate cancer cells while DAPI (blue) was used as a nuclear stain. Dashed box in merge represents area of magnification.

Conditioned media from the human bone metastatic cell line C42B and the rodent bone metastatic PaIII cell line were collected and supplemented with complete osteogenic supplement (OS) or to media supplemented with ascorbic acid (AA) and beta glycerol phosphate (BGP). Conditioned media from both cell lines added to either OS or AA/BGP supplemented media significantly increased the amount of osteogenesis in MSCs compared to controls as measured by alizarin red staining. (Figure 21)
Figure 18-1: Bone marrow-derived MSC effects on prostate cancer cells.

a. Representative images of α-Smooth muscle actin (SMA) staining of human and rodent bone metastatic prostate cancer. 

b. MSC migration to prostate cancer cell conditioned media. Number of hematoxylin and eosin stained MSCs per filter (MC No./Filter) were counted after 6 hours of incubation. Representative photomicrographs of fields of view are shown.

c. Direct co-culture of MSCs and PAIII prostate cancer cells at various ratios of PAIII:MSC. Values calculated as percentage of respective PAIII controls seeded at the same density (% Control). Growth was determined by luminescence assay and relative light unit (RLU) measurement.

d. MSC conditioned media (CM) treatment of PAIII at varying ratios. Final concentration of serum was 10% for each condition.

e. Cleaved caspase-3 (arrow head) in PAIII cells treated for 6 hours with MSC CM (50% ratio). Arrow indicates full-length caspase-3. Etoposide (ETX; 50μM) was used as a positive control.
Figure 19-2: Bone marrow-derived MSC effects on prostate cancer cells.

f. MSC CM (50%) effects on prostate cancer cell line growth relative to untreated controls. Growth measurements were performed by MTT or luminescence assay. g. PAIII treated with MSC or osteoblast (MC3T3) CM. h. Prostate epithelia cells (PREC) treated with MSC CM. MTT absorbance (ABS) was used as a readout for cell growth. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) while NS denotes not significant.

MSCs suppress the growth of bone metastatic prostate cancer in vivo:

To examine the effects of MSCs on prostate cancer progression in bone, immunocompromised animals were intratibially injected with luciferase-expressing PAIII PCa cell (2x10^4; n=8) in the presence or absence of 1:1 ratio of mouse MSCs (2x10^4; n=8) to reflect our in vitro observations. A separate cohort of mice received MSCs alone (2x10^4; n=7). Contralateral limbs in each animal received sham injections that served as an internal baseline control. We have previously shown that the PAIII PCa model generates rapid mixed osteolytic/osteogenic responses over the course of ~15 days prior to breaching the cortical bone [316, 318, 319]. Using bioluminescence as a correlate of tumor growth over time, we noted that, similar to effects observed in vitro,
PAIII PCa growth was significantly suppressed by MSCs versus PAIII-alone cohort up to day 11 post-transplant (Figure 23 a).

**Figure 20**: Characterization of mouse isolated MSCs.

MSCs were characterized using FLOW cytometry for CD29 and SCA1 positivity and CD45 negativity. The MSCs were then differentiated into osteoblasts stained with alizarin red, adipocytes stained with oil red O and chondrocytes stained with Alcian Blue.

However, between day 11 and 14 we observed that the growth of the PAIII cancer cells co-injected with the MSCs rapidly accelerated (a 1,260% increase in RLU from day 11 to 14) compared to the PAIII alone cohort (166% increase in RLU over the same time
period) rendering the differences in tumor burden insignificant by day 14 (Figure 23 b and c).

**Figure 21:** Prostate cancer CM enhances osteogenesis of MSCs

MSCs were differentiated using no supplements, osteogenic supplement, or a combination of ascorbic acid and beta glycerol phosphate. The supplements were added to either control media or media supplemented with 50% prostate cancer conditioned media. a. PAIII CM or b. C42B CM. The amount of differentiation after 21 days was significantly increased in the presence of the prostate cancer CM. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) whereas NS denotes not significant.

Analysis of proliferative (pHistone-H3) and apoptotic (cleaved caspase-3) indices confirmed increased proliferation and decreased apoptosis in the PAIII+MSC cohort with no statistical differences noted at the time of clinical endpoint compared to the
PAIII-alone cohort (Figure 23 d, e). Analysis of MSC markers also demonstrated the persistence of the MSCs over time in the PAIII+MSC cohort (Figure 23 f).

X-ray analyses of cancer-associated bone disease revealed, as expected [316, 318], significant areas of tumor-induced osteolysis in PAIII-bearing tibia compared to sham controls. However, strikingly, osteolysis was not evident in the PAIII+MSC cohort (Fig. 16g). Consistent with this observation, there were significantly higher numbers of TRAcP-positive mature multinucleated osteoclasts at the tumor-bone interface in the PAIII cohort compared to the PAIII+MSC, MSC or sham cohorts (Figure 23 h). Conversely, histomorphometry analysis demonstrated significantly higher levels of bone volume in the PAIII+MSC group compared to the PAIII cohort, which likely reflects increased MSC differentiation into osteoblasts (Figure 23 i).

Figure 22-1: MSCs initially suppress prostate cancer growth in the bone microenvironment.

a, b. Prostate cancer growth (PAIII) overtime in the presence (1:1 ratio) or absence of MSCs (n=8/group). An MSC-alone group was also included as a control (n=7). Representative images of bioluminescence for each group at day 11 time point are shown. Graphs illustrate collected RLUs over time for each group thru days 11 and 14. b. Analysis of RLU values at day 11.
Figure 23-2: MSCs initially suppress prostate cancer growth in the bone microenvironment.

c. and 14 in the PAIII vs. PAIII+MSC group. d, e. Ex vivo analyses from study endpoint of proliferative and apoptotic indices using phospho-histone H3 (p-H3; red arrows; c) and cleaved caspase 3 (CC3; red, arrows, d), respectively. Pan-cytokeratin (green) was used to identify prostate cancer cells. f.
Representative images of smooth muscle actin staining (α-SMA; red) in tissues derived from the PAIII+MSC group. Pan-cytokeratin (pCK; green) was used to localize prostate cancer cells. Dashed box in merge represents area of magnification. G. X-ray analysis of cancer-induced bone destruction. Representative X-ray from PAIII group is shown with dashed box defining area of magnification. Arrows indicate areas of cancer induced bone destruction. The area of bone destruction was calculated as a percentage of total volume. H. The number of osteoclasts (TRAcP positive; red, multinucleated; arrows) per μm of bone was calculated in non-sequential sections derived from the PAIII and PAIII+MSC groups. I. Trabecular bone volume (BV) was measured via histomorphometry on non-sequential H&E multiple sections derived from each group and calculated as a percentage of total volume. Representative gross H&E images are illustrated from the PAIII and PAIII+MSC group. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01) whereas NS denotes not significant.

Chronic MSC exposure selects for apoptosis resistant prostate cancer:

In vitro, we observed that some sensitive PCa cells treated with MSC CM persisted even after 24 hours of exposure to CM and were able to form colonies. To assess if MSCs promote the selection of apoptosis resistant subpopulations, we exposed parental PAIII PCa cells (F0) to MSC CM for 72 hours and allowed the surviving clones to grow out (F1). These cells underwent a consecutive round of MSC CM selection to yield a second MSC CM-educated population (F2). Cell growth analysis showed step-wise progressive enrichment of cancer cells resistant to growth inhibition by MSCs, F0<F1<F2 (Figure 25 a). Further, in direct co-culture experiments, with MSCs and cancer cells seeded at varying ratios, we observed an inhibitory effect on F0 parental cells but a proliferative effect on F2 MSC-selected cell lines (Figure 25 b). Consistent with this phenotype, immunofluorescence assays for cleaved caspase-3 demonstrated that apoptotic indices of F2 exposed to MSC CM were significantly lower than that of F0 cells (Figure 25 c). This effect was not limited to the PAIII PCa cells as chronic exposure to MSCs also selects for apoptosis resistant DU145 cells (Fig. 17d). We noted that both the F2 PAIII and F2 DU145 were also significantly more resistant to etoposide (ETX) induced apoptosis (Figure 25 c and e). To test if apoptosis resistance was solely due to
MSC-derived factors or was more generalizable, we also examined the sensitivity of F0 and F2 cells to the chemotherapeutic drug docetaxel. Notably, MTT assays established that the IC$_{50}$ docetaxel for F2 generated PAIII cells was 24-fold higher than that of the parental F0 cells (Fig. 17f). We further examined differences in the RNA profiles between the F0 and F2 PAIII and DU145 populations using RNA QuantSeq (Figure 28). Pathway and network analyses revealed that apoptotic and survival pathways were most impacted in the F2 prostate cancer cell populations underscoring that MSCs can drive the selection for apoptosis-resistant sub-populations of prostate cancer.

**Figure 24-1:** MSCs select for apoptosis resistant prostate cancer cell populations.

a. Growth of parental PAIIIs (F0) and MSC PAIII cell lines selected after one (F1) or two rounds (F2) of exposure to MSC conditioned media (50% concentration). Cell growth was calculated as a percentage of controls grown in the absence of MSC CM. b. Direct co-culture of F0 and F2 PAIIIs with MSCs at varying ratios (MSC:PAIII). Cell growth was calculated as a percentage of F0 and F2 PAIIIs seeded at equivalent numbers in the absence of MSCs. c. Immunofluorescence (IF) analysis of cleaved cspase-3 positivity
(green) in F0 and F2 PAIII cell lines treated for 6 hours with MSC CM. Graphs illustrate the number of cleaved caspase-3 positive cells as a ratio of total cell number (nuclear DAPI-blue) per multiple fields of view. Etoposide (ETX; 50μM) was used as a positive control.

Figure 25-2: MSCs select for apoptosis resistant prostate cancer cell populations.

d. e. MSC CM (50%) selection of apoptosis resistant DU-145 prostate cancer cells (F2) and the response to etoposide (ETX; 50μM). Cell growth was calculated by MTT assay with absorbance (ABS) at 490nm used as a correlate for cell number. F. IC50 curves of PAIII F0 and MSC selected F2 cells treated with docetaxel for 48 hours at a concentration range of 0 to 6.25nM. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) while NS denotes not significant.

Figure 26-1: RNA Quant profile of MSC educated PAIII and DU145 cell lines

a. b. Volcano plot showing log2 fold changes RNA profile between in the F2 PAIII (a) and DU145 (b) cells compared to their respective F0 controls.
Figure 27-2: RNA Quant profile of MSC educated PAIII and DU145 cell lines

a. b. Volcano plot showing log2 fold changes RNA profile between in the F2 PAIII (a) and DU145 (b) cells compared to their respective F0 controls. c. d. Network (c) and pathway (d) analyses of the RNA profiles in the F2 PAIII and DU145 compared to their respective F0 controls.
**Figure 28-3**: RNA Quant profile of MSC educated PAIII and DU145 cell lines

c. d. Network (c) and pathway (d) analyses of the RNA profiles in the F2 PAIII and DU145 compared to their respective F0 controls.

**MSCs accelerate prostate cancer progression in bone:**

To assess the effects of MSC selection on bone metastatic prostate cancer disease progression, immunocompromised animals were intratibially injected with luciferase-expressing F0 and F2 PAIII cell lines (2x10^4; n≥7) in the presence or absence of mouse MSCs (2x10^4; n≥7). Sham injected contralateral limbs in each animal served as an internal baseline control. Analysis of bioluminescence over time showed that the F2 cell line, rather than being suppressed by MSCs, grew at significantly faster rates compared to all other cohorts (Fig. 19a). Interestingly, F0 and F2 PAIII-derived tumors grew at comparable rates, suggesting that MSCs drive the accelerated growth effects of the F2 PAIII cell line in vivo. In accord with the phenotypes manifest in vivo, IHC analysis of α-SMA demonstrated the persistence of the MSCs in the cancer-bone microenvironment over the course of the studies (Fig. 19b). Ex vivo analyses of the proliferative indices of F0 versus F2 PAIII cells agreed with the in vivo bioluminescence
readouts, where there were significantly higher rates of proliferation in the F2 PAIII cells when grown with MSC compared to all other groups (Fig. 19c). Further, analysis of apoptotic indices showed significantly more apoptosis occurring in the F0 MSC treated group (Fig. 19d). Although there was little impact of MSC on parental versus F2 PAIII prostate cancer cells on associated bone disease, as measured by μCT and histomorphometry analyses (Fig. 19e and f), revealed significantly higher numbers of osteoclasts in the F2 PAIII-alone cohort (Fig. 19g). While this is indicative of higher rates of bone remodeling, it did not manifest as increased cancer-induced bone destruction.

Figure 29-1: MSC-selected prostate cancer cell growth is promoted rather than suppressed by the presence of MSCs.

a. Parental (F0 PAIII) and MSC selected (F2 PAIII) growth over time in the presence (1:1 ratio) or absence of MSCs (n≥8/group). Representative images of bioluminescence in each group are shown at day 11 time point. Graphs illustrate collected RLUs over time for each group. b. Representative images of smooth muscle actin staining (α-SMA; red) in tissues derived from the F0 and F2 groups in the presence or absence of MSCs. Pan-cytokeratin (pCK; green) was used to localize prostate cancer cells. Dashed
box in merge represents area of magnification. c. d. Ex vivo analyses from study endpoint of proliferative and apoptotic indices using phosphohistone H3 (pH3; red arrows; b) and cleaved caspase 3 (CC3; red, arrows, b) respectively. Pan-cytokeratin (green) was used to identify prostate cancer cells.

Figure 30-2: MSC-selected prostate cancer cell growth is promoted rather than suppressed by the presence of MSCs.

c. d. Ex vivo analyses from study endpoint of proliferative and apoptotic indices using phosphohistone H3 (pH3; red arrows; b) and cleaved caspase 3 (CC3; red, arrows, b) respectively. Pan-cytokeratin (green) was used to identify prostate cancer cells. e. μCT scan analysis of cancer-induced bone destruction. Representative μCT images of the trabecular bone are shown for the F0 and F2 PAIII group. The trabecular bone volume was calculated as a ratio to total volume analyzed (BV/TV). f. Trabecular bone volume (BV) was measured via histomorphometry on non-sequential H&E multiple sections derived from each group and calculated as a percentage of total volume. Representative gross H&E images are illustrated from the F0 and F2 groups. g. The number of osteoclasts (TRAcP positive; red, multinucleated; arrows) per μm of bone was calculated in non-sequential sections derived from each group. Asterisks denotes statistical significance (*p≤0.05, ****p≤0.0001) whereas NS denotes not significant.

Proteinase-K treatment and heat inactivation of the MSC CM revealed that a soluble protein was necessary for the observed apoptotic effects in co-culture with prostate
cancer cells (Figure 30 a). To identify potential apoptosis inducing factors cytokine arrays were performed, which revealed that Fas ligand (FasL), galectin 1 (GAL1), and interleukin-28 (IL-28) were specifically found in MSC-derived conditioned media (Figure 30 b). All 144 cytokines in the array were classified as either no expression, low expression or high expression (Table 2). A IL-28 is known to trigger apoptosis [299, 320-322] but has not been explored in the context of bone metastatic prostate cancer. Analysis of publicly available datasets revealed that IL-28, and its cognate receptors IL28Rα and IL10Rβ were expressed in prostate carcinoma compared to prostate glandular epithelium (Figure 35 a-c). We also demonstrated the presence of the IL-28Rα in pan-cytokeratin positive prostate cancer cells in human samples of bone metastatic prostate cancer (Figure 35). Next, we tested if PCa cells were sensitive to IL-28 induced apoptosis. Both F0 and F2 PAIII prostate cancer cells expressed IL-28Rα and IL-10Rβ, whereas MSCs expressed IL-28 (Figure 32 c). Notably, using recombinant IL-28, F2 PAIII cells were significantly more resistant to IL-28-mediated cell death, with an IC₅₀ >35-fold higher than that observed in the parental F0 cell lines (F0 IC₅₀ = 244pg/ml, F2 IC₅₀ = 9,145pg/ml; Figure 32 d). Further, addition of IL-28 neutralizing antibody to MSC CM, but not of isotype-matched IgG, ablated MSC-induced apoptosis of parental F0 PAIII cells (Figure 32 e). Similarly, efficient shRNA-directed knockdown of IL-28Rα expression in parental PAIII cells blocked MSC- and IL-28-induced apoptosis without affecting the growth of these cells (Figure 32 f and g). In contrast, MSC CM or recombinant IL-28 triggered rapid decreases in cell number in scrambled shRNA control cells (Figure 32 g). Similar findings were observed following shRNA-directed knockdown
of IL-28Rα in DU145 PCa cells (Figure 32 h, i), confirming the role of MSC-derived IL-28 in mediating the observed apoptotic effect.

**Figure 31-1: MSC-derived IL-28 directs PCa apoptosis**

MSC-derived IL-28 directs PCa apoptosis. a, PAIII growth (F0) in response to treatment with MSC CM, heat-inactivated (HI) MSC CM, or proteinase-K (PK) treated MSC CM. b, Cytokine Array of MSC CM. Black box indicates positive control (+ve), red box indicates IL-28. c, RT-PCR analysis of PAIII (F0 and F2) of IL28Rα, IL-10Rβ and IL-28 expression. Molecular weights in base pairs are shown. d, Growth of PAIII (F0) in MSC CM immune-depleted of IL-28 (MSC αIL-28). IgG was used as negative control (MSC αIgG).
IgG). Growth is expressed as a percentage of non-treated cells. e, Treatment of PAIII F0 and F2 cell lines with the indicated concentrations of recombinant IL-28 (rIL-28) for 48 hr. f, Growth of IL-28Rα silenced (sh-IL28R) and scrambled control (sh-SCR) compared to parental PAIII cell lines.

Figure 32-2: MSC-derived IL-28 directs PCa apoptosis
g, h, Control (sh-SCR) and IL-28Rα (sh-IL28R) PAIII and DU145 growth in MSC CM or rIL-28 as measured by luminescence assay and relative light unit (RLU) measurement or MTT assay. Asterisks denotes statistical significance (**p≤0.01, ****p≤0.0001) while NS denotes not significant.

Table 4: Expression of each of the cytokines on all three arrays after exposure to MSC CM.

Expression of each of the cytokines on all 3 arrays after exposure to MSC CM. Score 0 = array showed no expression 1=expression at high exposure only (low expression) 2=array showed expression at low exposure (high expression)

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Figure 33-1: IL-28Rα expression in human prostate cancer

a-c. Public database analysis of IL-28 a, IL-28Rα
Figure 34-2: IL-28Rα expression in human prostate cancer

a-c. Public database analysis of IL-28 a, IL-28Rα b, and IL10Rβ c, in human prostate gland (control) and primary prostate cancer tissue.
**Figure 35-3: IL-28Rα expression in human prostate cancer**

d. Representative images of IL-28Rα staining (red) in human samples of bone metastatic prostate (n=10; three representative images are shown). Pan-cytokeratin (pCK; green) was used to localize prostate cancer cells while DAPI (blue) was used as a nuclear stain. Dashed box in merge represents area of magnification.

**Development of MSC-derived resistance selects for IL-28-STAT3 signaling:**

Despite clear differences in sensitivity to IL-28-induced apoptosis, levels of IL-28Rα were similar in F0 versus F2 cell lines nor did we observe any differences in receptor induction in response to MSC CM over time (Figure 36). IL-28Rα induces phosphorylation of STATs via JAK/TYK kinase activation [323]. In the context of cancer, STAT1 is considered a tumor suppressor while STAT3 is often associated with tumor progression [324]. Consistent with previous publications [325], analysis of human bone metastatic specimens (n=10) demonstrated
phosphorylated STAT3 in pan cytokeratin positive prostate cancer cells (Figure 37). We therefore assessed the activity of STAT1 and STAT3 in our PCa cell models by monitoring their phosphorylation status. In independent experiments, we observed that at steady state, both PAIII and DU145 F0 cell lines had significantly higher levels of pSTAT1 (Tyr 701) compared to their respective F2 cell lines (Figure 38 a; 0 minute time point). Conversely, baseline pSTAT3 (Tyr 705) levels were reduced in the PAIII F2 versus F0 cells but comparable levels were noted in the DU145 cell lines (Figure 38 a; 0-minute time point). Notably, treatment with MSC CM resulted in select differences in pSTAT1 and pSTAT3 signaling in the F0 and F2 cell lines at 1, 5 and 10 minutes. Using quantitative STAT activity assays, we observed that MSC conditioned media significantly enhanced STAT1 activity in the PAIII and DU145 F0 cell lines compared to F2 response (Figure 38 c and d). Conversely, we identified that MSC CM significantly increased STAT3 activity in the PAIII F2 cell lines (Figure 38 c) but for DU145, MSC CM significantly lowered STAT3 activity in the F0 cells while having no effect on the F2 population (Figure 38 d). These data suggest that the MSC-selected apoptosis-resistant prostate cancer cells exhibit STAT3 predominantly to STAT1 signaling. Finally, in accord with its known pro-tumorigenic roles [326], siRNA-directed knockdown of STAT3 (Figure 38 e and g), reduced the growth of all PCa cells with the addition of MSC CM to the STAT3-silenced cells elicited no significant effect on cell viability (Figure 38 f and h).

**STAT3 inhibition suppresses the growth of MSC-educated prostate cancer cells:**

JAKs mediate IL-28Rα and IL-10β signal transduction [320], and several JAK inhibitors have entered the clinical setting [327].
Figure 36: IL-28Rα and IL-10Rβ expression in F0 and F2 PAIII and DU145 cell lines.  

a. IL-28Rα and IL-10Rβ expression in F0 and F2 PAIII and DU145 cell lines.  

B. STAT3 phosphorylation in PAIII parental (PAIII), scrambled control (sh-SCR) and IL-28Rα silenced (sh-IL28Rα) cell lines in
response to treatment with recombinant IL-28 (rIL-28; 25ng/ml) for 15 minutes. Actin was used a loading control. Molecular weights are shown in kDa. c-f. Realtime qPCR analysis of IL28Rα and IL10Rβ in F0 and F2 PAIII (c, d) and DU145 (e, f) cells lines in response to MSC CM at 1, 5, 10 and 60 minutes. Asterisk denotes statistical significance (*p≤0.05).

Figure 37: pSTAT3 localization in human bone metastatic prostate cancer specimens.

Representative images of pSTAT3 staining (red) in human samples of bone metastatic prostate cancer (n=10; five representative patients are shown). Pan-cytokeratin (pCK; green) was used to localize prostate cancer cells while DAPI (blue) was used as a nuclear stain. Dashed box in merge represents area of magnification. Arrows used to identify pSTAT3 staining in prostate cancer cells while arrow heads identify positive staining in the supporting bone stromal cells.

Treatment with the JAK1/JAK2 inhibitor ruxolitinib reduced STAT3 phosphorylation and impaired the growth of both F0 and F2 MSC-selected F2 PAIII and DU145 cells (Figure 40 a and Figure 27 a). Of note, in this system, ruxolitinib and S3I-201 elicited no significant effect on STAT1 activity (Figure 42 a).
Figure 38: Elevated STAT3 signaling in MSC-selected prostate cancer cell lines.

a, b. pSTAT1 and pSTAT3 levels at baseline and in response to MSC CM (50%) over a 10 minute (min) period in PAIII (a) and DU145 (b) parental (F0) and MSC selected (F2) cell lines. Molecular weights are shown in kDa. Actin was used as a loading control. c, d. STAT1 and STAT3 activity in the PAIII (c) and DU145 (d) was measured in response to MSC CM for 30 minutes. e, f. STAT3 was silenced (si-STAT3) in PAIII (e) and DU145 (f) F0 parental and F2 MSC-selected cell lines and the resultant impact on STAT3 activity was measured. Blots show total STAT3. g, h. The effect of STAT3 silencing on PAIII (g) and DU145 (h) cell growth in the presence or absence of MSC CM compared to control treated cells using luminescence assay and relative light unit (RLU) measurement or MTT assay. Molecular weights are shown in kDa. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) while NS denotes not significant.
We next focused on specific inhibition of STAT3. Translational efforts in this area have proven challenging, but the development of STAT3 inhibitors is important given high levels of pSTAT3 in advanced cancers [324-326, 328]. In collaborative efforts, we previously developed S3I-201, an inhibitor that prevents STAT3 dimerization, and demonstrated its efficacy in treating breast cancer growth in vivo with no noted toxicities [302]. Notably, treatment with S3I-201 selectively impaired the growth of the MSC-selected F2 PAIII and DU145 cell lines with little effects on F0 parental cell lines (Figure 26 b). The specificity of S3I-201 was confirmed in STAT3-silenced F2 cell lines, where treatment led to no further decrease in cell growth than that provoked by knockdown of STAT3 alone (Figure 26 c). To test the sensitivity of the F2 generated prostate cancer cell lines to STAT3 inhibition, we assessed the efficacy of the S3I-201 inhibitor in vivo. Mice were intratibially injected with luciferase expressing F0 and F2 DU145 cell lines and randomized after 7 days into vehicle control (n=10) or S3I-201 groups (n=10). Over time we observed that S3I-201 significantly reduced the intraosseous growth of the F0 and F2 groups compared to their respective controls (Figure 26 d). Normalization to controls at study endpoint further demonstrated that S3I-201 was more effective in reducing the growth of the F2 DU145 compared to the F0 DU145 population (Figure 26 e). Ex vivo analyses of the injected tibia revealed that S3I-201 significantly inhibited the proliferation of the F0 and F2 PCa cells (pHistone H3) while augmenting the apoptosis index (cleaved caspase-3), particularly in the F2 group (Figure 26 f, g). Consistent with reduced cancer growth, S3I-201 also protected against cancer associated bone disease as measured by μCT, X-ray, histomorphometry and TRAcP staining (Figure 44). S3I-
201 also effectively limited the growth of F2 PAIII PCa cell lines \textit{in vivo} and significantly suppressed proliferation (pHistone H3) (Figure 46). Despite decreased tumor growth, we detected no differences in cancer-associated bone disease, but this might be due to the rapid nature of the PAIII model (2 weeks) compared to the DU145 model (6 weeks). Importantly, however, our findings establish that MSC-selected apoptosis-resistant F2 prostate cancer cells are highly sensitive to STAT3 inhibition \textit{in vivo}.

\textbf{Figure 39-1}: STAT3 inhibition impairs the growth of MSC-selected prostate cancer \textit{in vitro} and \textit{in vivo}.

\textbf{a, b}. Parental (F0) and MSC-selected (F2) cell lines treated with vehicle control (Control) or the JAK2 inhibitor ruxolitinib (RUX)/STAT3 inhibitor (S3I-201) for 24 hr. \textbf{c}. F0 and F2 DU145 control (scr-siRNA) or STAT3 silenced (si-STAT3) cells treated with vehicle or S3I-201 for 24 hr. \textbf{d}. F0 and F2 DU145 growth over time in the presence or absence of STAT3 inhibitor, S3I-201 (n=10/group). Representative images of
bioluminescence in each group are shown at day 35 time point. Arrow and dashed line represent time of treatment initiation. Graphs illustrate collected RLUs over time for each group.

**Figure 40-2:** STAT3 inhibition impairs the growth of MSC-selected prostate cancer *in vitro* and *in vivo*.

d. F0 and F2 DU145 growth over time in the presence or absence of STAT3 inhibitor, S3I-201 (n=10/group). Representative images of bioluminescence in each group are shown at day 35 time point. Arrow and dashed line represent time of treatment initiation. Graphs illustrate collected RLUs over time
for each group. e. S3I-201 effect on F0 and F2 DU145 at day 42 normalized to respective controls. f, g. Ex vivo analyses from study endpoint of proliferative and apoptotic indices using phosphohistone H3 (pHH3; red arrows; f) and cleaved caspase 3 (CC3; red, arrows, g) respectively. Pan-cytokeratin (green) was used to identify prostate cancer cells. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) whereas NS denotes not significant.

**Figure 41-1:** STAT3 phosphorylation status in response to ruxolitinib and S3I-201.

**a-d.** STAT3 and STAT1 phosphorylation and activity was assessed in response to ruxolitinib (Rux; 100nM) and S3I-201 (100 μM) treatment overnight in F0 and F2 PAIII (a, b) and DU145 (c, d) cell lines. Subsequently, MSC CM was added to each condition for 30 minutes prior to lysis and analysis. Actin was used as a positive loading control. Molecular weights are shown in kDa. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001) whereas NS denotes not significant.
Figure 42-2: STAT3 phosphorylation status in response to ruxolitinib and S3I-201.

a-d. STAT3 and STAT1 phosphorylation and activity was assessed in response to ruxolitinib (Rux; 100nM) and S3I-201 (100 μM) treatment overnight in F0 and F2 PAIII (a, b) and DU145 (c, d) cell lines. Subsequently, MSC CM was added to each condition for 30 minutes prior to lysis and analysis. Actin was used as a positive loading control. Molecular weights are shown in kDa. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001) whereas NS denotes not significant.
Figure 43-1: STAT3 impacts prostate cancer associated bone disease in the F0 and F2 DU145 model.

a. μCT scan analysis of cancer-induced bone destruction. Representative μCT images of the trabecular bone are shown for the vehicle alone and S3I-201 F0 and x. The trabecular bone volume was calculated as a ratio to total volume analyzed (BV/TV).

b. Faxitron X-ray analysis of cancer-induced osteolytic lesions. Representative images of the X-rays are shown for each group. The size of the tumor volume as measured by the area of the lytic lesion (TuV) was calculated as a ratio to total volume analyzed (TuV/TV).
**Figure 44-2:** STAT3 impacts prostate cancer associated bone disease in the F0 and F2 DU145 model.

c. Trabecular bone volume (BV) was measured via histomorphometry on non-sequential hematoxylin and eosin (H&E) multiple sections derived from each group and calculated as a percentage of total volume. Representative gross H&E images are shown for the vehicle and S3I-201 F0 and F2 groups. d. The number of osteoclasts (TRAcP positive; red, multi-nucleated; arrows) per μm of bone was calculated in non-sequential sections derived from each group. Representative gross TRAcP images are shown for the vehicle and S3I-201 F0 and F2 groups. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) whereas NS denotes not significant.
Figure 45-1: STAT3 inhibition impairs the growth of MSC-selected prostate cancer in vitro and in vivo.

a. F0 and F2 PAIII growth over time in the presence or absence of STAT3 inhibitor, S3I-201 (n≥8/group). Representative images of bioluminescence in each group are shown at day 11 time point. Arrow and dashed line represent time of treatment initiation. Graphs illustrate collected RLUs over time for each group. b. S3I-201 effect on F0 and F2 PAIII at day 11 normalized to respective controls. c. d. Ex vivo analyses from study endpoint of proliferative and apoptotic indices using phosphohistone H3 (pHH3; red
arrows; b) and cleaved caspase 3 (CC3; red, arrows, c) respectively. Pan-cytokeratin (green) was used to identify prostate cancer cells.

**Figure 46-2:** STAT3 inhibition impairs the growth of MSC-selected prostate cancer *in vitro* and *in vivo*.

e. μCT scan analysis of cancer-induced bone destruction. Representative μCT images of the trabecular bone are shown for the vehicle alone and S3I-201 F0 groups. The trabecular bone volume was calculated as a ratio to total volume analyzed (BV/TV). f. Trabecular bone volume (BV) was measured via histomorphometry on non-sequential hematoxylin and eosin (H&E) multiple sections derived from each group and calculated as a percentage of total volume. Representative gross H&E images are shown for the vehicle and S3I-201 F0 groups. g. The number of osteoclasts (TRAcP positive; red, multi-nucleated; arrows) per μm of bone was calculated in non-sequential sections derived from each group. Representative gross TRAcP images are shown for the vehicle and S3I-201 F0 groups. Asterisks denotes statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001) whereas NS denotes not significant.
**Discussion:**

Bone metastatic prostate cancer remains incurable and affects the majority of men diagnosed with recurrent castrate resistant disease. These metastatic lesions are associated with extensive bone remodeling, which generates factors that promote the growth, survival and persistence of prostate cancer cells in the face of applied systemic ADT and chemotherapies [229, 290, 329]. Understanding how osteoblasts and osteoclasts mediate these effects has led to the development of therapies that target the microenvironment such as the RANKL-binding monoclonal antibody denosumab and nitrogen-containing bisphosphonates that block osteoclast formation and activity [290].

While effective in preventing skeletal pathologies such as fracture, these therapies are, unfortunately, largely palliative. The heavy infiltrates of osteoblast progenitor MSCs in bone metastatic prostate cancer we observed is not surprising considering the bony nature of these lesions, [313, 330] and that prostate cancers are known to drive MSCs into an osteoblastic phenotype [289, 312, 313, 331]. However, the dynamic effects of MSCs in promoting apoptosis and hence the selection for therapy resistance disease via MSC-derived IL-28 was unexpected, where MSC-derived IL-28-induced apoptosis drives the selection for broadly resistant subpopulations that are growth stimulated rather than repressed by MSCs. Further, the switch from apoptosis sensitivity to resistance was accompanied by a shift in STAT1 to STAT3 signaling that we have shown represents a vulnerability. Our *in vivo* studies demonstrated that STAT3 inhibition with S3I-201, was effective in significantly reducing the growth of MSC-selected prostate cancer. Our working hypothesis is that upon entry into the bone
marrow, metastatic prostate cancer cells interact with resident MSCs, resulting in the elimination of a majority of the cancer cells being eliminated. Over time however, MSCs eventually select for prostate cancer cells that are refractory to IL-28-induced apoptosis and that exhibit increased STAT3 signaling. Interestingly, we found that the MSC-selected prostate cancer cells are also cross-resistant to chemotherapies such as etoposide and docetaxel suggesting that STAT3 inhibition could be a viable route to resensitizing bone metastatic prostate cancer that is refractory to chemotherapy.

The tumor-promoting properties of MSCs revealed herein are largely in accord with studies establishing that MSCs contribute to, and in some cases are necessary for, tumor progression. For example, in breast cancer, intratumoral MSCs express chemokine ligand 5 (CCL5) that promotes invasion and metastasis [255], and co-inoculation with MSCs promotes progression and metastasis of both osteosarcoma and ovarian cancer [256, 332]. Mechanisms ascribed to these effects include angiogenesis and protection from hypoxia via the secretion of vascular endothelial growth factor (VEGF), immune suppression/modulation, the suppression of apoptosis, and the induction of the epithelial to mesenchymal transition (EMT) program [275, 276]. Consistent with the positive effect of MSCs on cancer cell growth, we have shown here that bone-marrow derived MSCs enhance the proliferation of a subset of metastatic prostate cancer cell lines such as C4-2B, which of notably is derived from in vivo LnCAP bone metastases and exhibits endogenously elevated levels of STAT3 activity [333]. Conversely, MSCs can have tumor suppressive effects in hepatoma and glioma where they induce cell-cycle arrest and apoptosis [334]. MSC-derived interferons (IFNs) have been shown to trigger apoptosis in MCF-7 breast cancer cells via activation of STAT1.
Notably, a similar mechanism appears manifest in prostate cancer cells that are vulnerable to MSC-derived IL-28 induced apoptosis via STAT1 activation. As documented here, IL-28 joins a cast of other cytokines that activate STAT3, including IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM) and IL-31 [336, 337]. STAT3 has clear roles in driving cancer progression and therapy resistance [324, 325, 338-340] but rather little has been described regarding the tumorigenic roles of IL-28. In a mouse model of B16 melanoma progression, IL-28 reduces tumorigenicity but how this occurs is not clear and could reflect effects on immune surveillance [341]. This remains an important area for future investigation in bone metastatic prostate cancer, where syngeneic cell lines in immunocompetent animals (TRAMP-C3, Myc-CAP, RM1) will allow for the study of MSCs and IL-28 on infiltrating immune cells.

In addition to IL-28, our cytokine array analysis also revealed the presence of additional apoptotic factors in the MSC CM, such as FasL. Indeed, release of FasL via matrix metalloproteinase-7 (MMP-7) has been shown to be responsible for prostate epithelial cell apoptosis during involution of the organ subsequent to castration [342]. This phenomenon has also been demonstrated in the mammary gland where expression of a MMP-7 transgene in mammary epithelium augments apoptosis and involution at weaning but eventually provokes the development of hyperplasia in multiparous mice [343]. Further, chronic exposure of breast cancer cells to soluble FasL selects for apoptosis resistant subpopulations [344]. However, in our models, PAIII are sensitive to FasL-induced apoptosis (Figure 47), immunodepletion of IL-28 completely abrogates the apoptotic effects of MSC CM (Figure 32 e).
MSC-selected apoptosis-resistant prostate cancer cells exhibit elevated pSTAT3, consistent with the hyperactivation of STAT3 signaling observed in many human cancers where that correlates with poor prognosis and resistance to chemotherapy and radiation therapy [339, 345]. In bone metastatic prostate cancer, IHC analyses have revealed that the majority of cases studied are positive for STAT3, and kinome profiling have shown elevated activity of JAK2, which phosphorylates STAT3 at Tyr705 resulting in head to tail dimerization, translocation to the nucleus and binding to the promoters of target survival genes such as BCL-xL and survivin [346]. Here, we have shown preferential STAT3 activity in MSC/IL-28-resistant prostate cancer that confers resistance to chemotherapies used to treat bone metastatic prostate cancer. Since there is no change in the level of IL28Rα or IL10Rβ, it is not clear at this juncture what causes the termination of STAT1 phosphorylation in the MSC-educated prostate cancer cells. STAT inactivation can be controlled by a number of mechanisms that might be altered between the F0 and F2 populations including protein tyrosine phosphatases that depending on kinetics and cellular compartmentalization may preferentially dephosphorylate specific STATs [347-349]. Suppressors of cytokine signaling (SOCS) and protein inhibitors of activated STATs (PIAS) can also act as inhibitors of STATs or direct them for protein degradation [347]. For example, STAT-interacting LIM protein (SLIM) acts as a ubiquitin E3 ligase and can direct STATs, in particular, STAT1, for proteasomal degradation [350]. Further, STAT3 itself can bind to STAT1 and prevent the transcription of STAT1 target gene suggesting that STAT3 activity in the F2 cells could further limit STAT1 activity [351]. RNA QuantSeq analysis comparing the MSC educated F2 cell lines to their parental counterparts also demonstrated that genes such
as SPRY2 (a negative regulator of interferon signaling and IFN inducible genes) were significantly downregulated in MSC educated cells. Further, bioinformatic analyses of the most downregulated pathways and networks in the MSC educated PAIII and DU145 F2 populations are; apoptosis regulated by mitochondrial proteins and apoptotic mitochondria respectively (Figure 28). Interestingly, STAT3 has been shown to accumulate in the mitochondria [352], prevent mitochondrial mediated apoptosis and is linked to enhanced survival and drug resistance [353, 354]. We should also note that deletion of STAT3 and PTEN in genetically engineered mouse models demonstrated a protective effect on prostate cancer progression and soft tissue metastasis (liver and lungs) [340]. Further, STAT3 expression was detected in only ~40% of metastases but the location of the metastases was not obvious. The tumor microenvironmental context of the metastases can have profound effects on cancer cell behavior. Given that other reports have reported high STAT3 positivity in bone metastatic prostate cancer [325] and that the majority of prostate cancer in humans metastasizes to the skeleton, we believe that STAT3 may be an important regulator of prostate cancer progression, specifically in bone. Importantly, STAT3 is also revealed here to be a targetable vulnerability that disables the growth of bone metastatic PCa, suggesting that STAT3 inhibitors such S3I-201 [302], which appears well-tolerated in pre-clinical studies, and have potential in the prostate cancer clinic. Alternatively, FDA-approved JAK2 inhibitors such as ruxolitinib could be considered for the treatment of metastatic CRPC patients, yet to date, ruxolitinib has produced only very modest effects in phase II clinical trials (NCT00638378), suggesting more specific targeting of JAK2 or better delivery methods are required to realize the therapeutic potential of JAK2 inhibition.
In conclusion, we have shown that bone marrow-derived MSCs drive the emergence of apoptosis resistant subpopulations of prostate cancer cells via the chronic exposure to MSC-derived IL-28 and that this is associated with increases in STAT3 activity that are necessary for the maintenance of bone metastatic PCa. Our findings also indicate that the application of STAT3 inhibitors may resensitize prostate cancer cells to chemotherapy and that, given the role of STAT3 activity in the progression of a wide range of cancers, the mechanism described herein may have broad applicability to other skeletal malignancies and/or metastases.

Materials and Methods:

**Cell lines, culture and animals:**

LNCaP (Cat # CRL-1740), DU145 (HTB-81), MC3T3 (CRL-2594), RWPE-1 (CRL-11609), V-CAP (CRL-2876), 22RV1 (CRL-2505) MyC-CaP (CRL-3255) cell lines were purchased from the ATCC. PC3-2M cells were purchased from Perkin Elmer, PrEC prostate epithelial cells (CC-2555) and human MSCs (PT-2501) were purchased from Lonza while PAIII cells [27] and C4-2B [67] were kindly donated. All cell lines were periodically mycoplasma tested (CUL001B, R&D Systems) and short tandem repeat (STR) verified at the Moffitt Clinical Translational Research Core. Cell lines were passaged in recommended culture medium supplemented with 10% fetal calf serum (FCS). Isolation and culture methods for mesenchymal stem cells (MSCs) were adapted from previously published protocols [355].
**Figure 47: MSC-derived FasL promotes PCa apoptosis.**

a. Cytokine Array of MSC CM. Positive control and levels of FasL and IL-28 are illustrated. Cytokine Array of MSC CM. Black box indicates positive control, red box indicates IL-28. b. PAIII response to increasing concentrations of FasL for 48 hours. Anti-HA alone was used as a control. c, d. F0 and F2 PAIII and DU145 cell lines treated in the presence or absence of recombinant FasL (10ng/mL) for 48 hours. e. Immunofluorescence (IF) analysis of cleaved caspase-3 positivity (green) in F0 (d) and F2 (d) PAIII cell lines treated for 6 hours with rFasL. Graphs illustrate the number of cleaved caspase-3 positive cells as a ratio of total cell number (nuclear DAPI-blue) per multiple fields of view. Etoposide (ETX; 50μM) was used as a positive control. Asterisks denotes statistical significance (**p≤0.01, ****p≤0.0001) whereas NS denotes not significant.

Briefly, hind limbs were collected from tumor-naïve 4–6-week-old male C57/BL6 Rag2−/− mice in sterile PBS. Following removal of muscle tissue, epiphyses were removed, and bone marrow flushed three times with sterile PBS to deplete the hematopoietic cells. Flushed bones were then cut into 1–3 mm chips, digested with 1 mg/mL collagenase II (Invitrogen) in α-MEM with 15% FBS, and shaken at 150 RPM for 1 hour at 37 °C. Digested bone fragments were grown in 6-well tissue culture plates in α-MEM with 15% FCS. Medium was changed every 3 days. For direct co-culture
experiments, cancer cells expressing luciferase were cultured with murine MSCs at multiple ratios seeded for a total density of $2 \times 10^4$ in 48-well plates. Co-cultures were incubated for 48 hours, and PAIII growth was measured by bioluminescence using the Promega Luciferase Assay System (E1500) per manufacturer's instructions. For analyses assessing the growth of cancer cell lines in response to MSC CM, MTT assays were used. Prostate cancer cell lines were plated in 96-well plates at a density of $1 \times 10^4$ cells/well and treated with MSC conditioned media. Cell viability was measured at 48 hours by the MTT assay per manufacturer's protocols (CellTiter 96, #G3582, Pierce) by measuring absorbance at 490nm after 4 hours of incubation at 37°C.

**MSC characterization by FACS analysis:**

The Mouse Mesenchymal Stem Cell Multi-Color Flow Kit (R&D Cat # FMC003) was used according to manufacturer’s protocol. Isolated MSCs were assessed by flow cytometry for CD29 and SCA-1 positivity and CD45 negativity. For FACs analysis $1 \times 10^6$ MSCs were suspended in flow cytometry staining buffer. For each marker 90μl of the cell suspension were transferred to 5ml flow tubes and incubated for 30 minutes with each antibody or isotype control. Cells were then washed twice in 2 ml of flow cytometry staining buffer and resuspended in 200μl of the same buffer for analysis.

**Differentiation assays:**

Osteogenic differentiation and staining: MSCs grown to confluency before treatment with 20X murine osteogenic supplement (R&D Cat# CCM009) added to normal growth medium and changed every 2-3 days for 21 days. Negative controls were grown in normal growth medium. Cells were then stained with Alizarin Red (Fisher scientific Cat
Cells were washed gently 3 times in PBS and fixed for 15 minutes using 10% buffered formalin. Fixed cells were washed 2 times with ddH$_2$O and then stained in the dark for 45 minutes in 2% alizarin red solution. (Note pH is critical for 2% Alizarin Red solution pH 4.2-4.3 adjusted using 10% ammonium hydroxide). Cells were washed gently 4 times with ddH$_2$O and air dried before images were acquired. For adipogenic differentiation and staining: MSCs were grown to confluency and then treated with 100X Adipogenic supplement (R&D Cat# CCM011) in normal growth medium or negative control in normal growth medium changed every 2-3 days for 21 days. Cells were stained with Oil Red O. (Sigma Cat# 01391). Cells were washed with PBS and then stained using 1-part Oil Red O 1-part water for 15 minutes. Cells washed 3 times with ddH2O and imaged for red oil droplets. For chondrogenic differentiation and staining: MSCs were trypsinized and counted 1.5X10$^5$ cells were spun down at 200g for 5 minutes in 6 15 ml conical tubes. The media was then replaced with media containing 100X chondrogenic supplement (R&D Cat# CCM006) or just base media in triplicate. The caps of the tubes were loosened and incubated at 37°C. After 2 days the cells formed small spheres and the media was replaced every 2-3 days for 28 days. The pellets were then washed in PBS and embedded in paraffin for histology. Consecutive slides were H&E stained and the second slide stained with Alcian Blue (Sigma Cat # B8438). For Alcian Blue staining slides were dewaxed in xylenes rehydrated to water and stained for 30 minutes in Alcian Blue solution. Slides were then washed in running tap water for 2 minutes followed by a rinse in H$_2$O. Slides were then dehydrated to 100% ethanol and cleared in xylenes before mounting cover slips with permount and imaging.
**Migration assay:**

Cells were serum starved for 2 hours before trypsinizing and seeding ($2 \times 10^5$ cells) into upper chambers of 24-well Transwell membrane assay system (Corning). Lower chambers were prepared either 650µl of either serum free, 1% serum or MSC CM. All conditions were performed in triplicate and incubated for 5 hours at 37°C. After incubation, upper chambers were rinsed in diH$_2$O water followed by 1X PBS and fixation in methanol at -20°C for 20 minutes. The chambers were then rinsed in water followed by 1X PBS, and non-migrated cells removed by gentle scrubbing with cotton tip applicators. After rinsing in diH$_2$O, membranes were stained with hematoxylin and dehydrated with 100% ethanol. The membranes were air dried dry and excised using a scalpel before mounting on slides with Permount (Fisher Cat # SP15-100). Three fields of view from each membrane were acquired using brightfield microscopy and the number of migrated cells per field counted.

**Intratibial tumor studies:**

Mice were purchased from Jackson Laboratory. All animal experiments were performed with IACUC approval (R1762, CCL) and in accordance with the guidelines set forth in the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. Six-week old male Rag2-/- mice were intratibially injected with luciferase-expressing PAIII or murine MSCs ($2 \times 10^4$ cells 20µl of sterile saline), or PAIIIs and murine MSCs 1:1 for a total of ($4 \times 10^4$ cells 20µl of sterile saline). Six-week old NSG mice were intratibially injected with luciferase-expressing DU145 cells ($5 \times 10^5$ in 20ul of sterile saline). Contralateral limbs were injected with saline to control for injury induced changes. For S3I-201 in vivo studies, tumors were allowed to establish for 3
days before randomizing groups for treatment with S3I-201 (5mg/kg) or sterile PBS administered by intraperitoneal injections in 200-μl volumes every other day. Bioluminescence for all studies was measured twice weekly as a correlate of tumor growth (IVIS™ Perkin Elmer). Mice that showed tumor growth outside of the bone compartment were excluded from all analyses.

**Cell line conditioned media collection and treatment:**

MSCs were cultured in T-75 cell culture flasks until 80-90% confluent. Each flask was rinsed three times with sterile 1X PBS before adding 5 mL of serum free medium. After incubating for 24 hours, conditioned media (CM) was centrifuged at 4,000g for 5 min, transferred to a new tube, and stored at 4°C. Fresh CM was collected weekly and stored at 4°C. To test if the MSC-derived factor responsible for prostate cancer apoptosis was a soluble protein, MSC CM and matched control serum free αMEM were either heat inactivated at 95°C for 30 minutes or treated with 100μg/ml proteinase K followed by heat inactivation 95°C for 30 minutes. PAIII cells were seeded at 3x10^4 cells per well and treated with each respective media type for 24 hr. Cell growth was analyzed by luminescence assay (Promega Luciferase Assay System, Cat # E1500).

For IL-28 neutralization studies; PAIII cells were seeded in white wall, solid bottom 96-well plates (5x10^4 cells/well) and incubated for 24 hours before treatment with either MSC CM or serum free media control containing either 10μg/ml of neutralizing antibody (R&D, AF1789) or normal goat IGG (R&D, AB108C). Cells were incubated for 48 hr, and growth was analyzed using the Promega Luciferase Assay System, Cat# E1500 per the manufacturer’s protocols.
For docetaxel, recombinant IL-28, JAK2 inhibition (ruxolitinib) and STAT3 inhibition (S3I-201) parental (F0) or MSC-selected prostate cancer cells (F2) cells were seeded (5X10^3) in 96-well plates and treated as follows. Docetaxel; 0, 0.0625, 0.125, 0.625, 1.25, 2.5, and 6.25 nM for 48 hours. Recombinant IL-28 (R&D mIL-28B, Catalog #1789-ML); 0, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128ng/ml. Ruxolitinb; 0, 0.0001, 0.001, 0.01, 0.1, 1, and 10 μM. S3I-201 0, 0.1, 0.5, 1, 5, 10, 25, 50, 100, and 150μM. Cell growth was assayed via luminescence or MTT assay.

**Ex vivo bone analysis:**

Tibias were collected and fixed in 10% formalin for 24-48 hours and then transferred to 50% ethanol. Radiographic images (Faxitron, X-ray Corp) were obtained using energy of 35kVp and an exposure time of 8 milliseconds. The spatial resolution is 10 lp/mm (48μm). The tumor volume (TuV) was calculated as a function of the total tissue volume (TV) of the tibial medullary canal using ImageJ software. For μCT analysis, the proximal tibia metaphyses were scanned (μCT-40; Scanco Medical). An evaluation of trabecular bone structural parameters was performed in a region that consisted of 1mm starting at 500μm from the growth plate. A three-dimensional cubical voxel model of bone was built, and calculations were made for relative bone volume per total volume and trabecular number. After X-ray and μCT analysis, tibias were decalcified (14% EDTA, pH 7.4, 3 weeks), processed, and paraffin embedded.

**Immunofluorescence:**

For paraffin embedded tissues, slides were dewaxed and rehydrated to water. Antigen retrieval was performed by heat using a pressure cooker for 5 minutes in 1X Tris EDTA.
pH 8.0. Proteinase K antigen retrieval was used for αSMA staining (7.5 minutes at room temperature 5mL 2M Tris pH 7.5, 5 mL 0.2M EDTA, 190 mL ddH2O and 400 µL of 10mg/mL proteinase K). Slides were blocked in 10% goat serum in 1X TBS for 1-hour room temperature. Primary antibodies (Pan Cytokeratin, Sigma-Aldrich Cat # C2562, 1:200 dilution; Phospho-Histone H3, Cell Signaling Cat #9701L, 1:200 dilution; Cleaved Caspase 3, Cell Signaling Cat #9661S, 1:200 dilution; Alpha Smooth Muscle Actin (1A4), Cell Signaling Cat # 48938, 1:200 dilution IL-28Rα, Bioss, Cat # ABIN1387718) Cell Signaling Cat#9145 pSTAT3 Tyr705 (D3A7) 1:100 dilution, abcam Cat #ab92574 CD90/Thy1 [EPR3132] 1:100 and R&D Cat# MAB2636 Mouse/Rat Nestin 1:50 were diluted in 10% normal goat serum (Vector Laboratories Cat # S-1000) and incubated overnight at 4°C in a humidified chamber. After 3 washes in 1X TBST followed by 1 wash in 1X TBS, secondary antibodies (Alexa FluorTM Goat Anti Rabbit 568, (Thermo Fisher Scientific #A-11011); Alexa Fluor Goat Anti Mouse 488, (Thermo Fisher Scientific #A32723) were incubated at a 1:1,000 dilution in 10% normal goat serum for 1 hour at room temperature. Slides were washed three times in 1X TBS and mounted using Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, # H-1200). Slides stored in the dark until image acquisition. At least 3 representative images of tumor sections were acquired and analyzed with Image J.

For in vitro immunofluorescent analyses, PAIII cells were seeded into 8-well chamber slides (LAB-TEK Cat #154534) at 2x10^4 cells per well and cultured overnight before treatment with either 50% MSC CM or DMEM 5% FBS or 100nM etoposide for 5 hours. Cells were then rinsed with PBS and fixed in 4% PFA at room temperature for 20 minutes. Fixed cells were then blocked for 30 minutes at room temperature in antibody
diluting buffer (2% BSA, 0.1% Triton X-100 in PBS). Primary antibodies (Cleaved Caspase 3, Cell Signaling Technology, #9661S), 1:400 dilution in antibody diluting buffer; Rabbit IgG Isotype Control, Thermo Scientific #31235) were incubated at room temperature for 30 minutes. Cells were then washed 3x in PBS and incubated with secondary antibody (Alexa FluorTM Goat Anti-Rabbit 488, Invitrogen #A-11034, 1:1,000 dilution in antibody diluting buffer) for 30 minutes at room temp in the dark. After washing 3x in PBS, culture chambers were removed and the slides mounted with Vectashield Antifade Mounting Medium with DAPI(Vector Laboratories, # H-1200). Mounted slides were stored in the dark at 4°C until microscopic analysis. Three representative images were acquired at 40X magnification and the ratio of cleaved caspase positive to negative cells calculated with ImageJ.

**H&E and TRAP staining:**

Formalin-fixed paraffin-embedded tissue sections cut5 μm thick were mounted on microscope slides and stained with hematoxylin and eosin for histological analyses. For TRAcP staining, slides were incubated in buffer (112 mM anhydrous sodium acetate, 49 mM dibasic dehydrate sodium tartrate, 0.28% glacial acetic acid) containing 1% naphthol-phosphate substrate (2% napthol AS-BI phosphate in 2-ethoxyethanol) for 1 hour at 37°C. Slides were then transferred to buffer containing 250 μl of 5% pararosaniline dye in 2 N HCl and 250 μl of 4% sodium nitrite at 37°C and monitored for development of red stained osteoclasts. Slides were rinsed in H2O, counterstained with hematoxylin, and aqueously mounted. The number of bone-lining, multi-nucleated (>3 nuclei per cell), TRAcP positive osteoclasts was quantified from multiple sections.
**Immunoblotting:**

Cells were lysed with RIPA (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris, pH 8). Protein concentration was determined by BCA (Pierce, Waltham, MA, USA; #23225). Blots were blocked in 5% BSA for 1 hour followed by primary antibody. All primary antibodies were purchased from Cell Signaling Technology (Cleaved Caspase 3 #9661S, pSTAT1 Tyr701 #7649S, pSTAT1 Ser727 #9177, pSTAT3 Tyr705 (D3A7) #9145, STAT3 (D3Z26) #12640S, STAT1 #9172S, pJAK2 Y1007/1008 # 3776S), Santa Cruz (IL-10Rβ, Cat# sc271969) or Abcam (IL-28Rα, Cat# ab83865). Primary antibodies were diluted 1:1,000 in blocking solution +0.1% Tween-20, and were incubated overnight at 4°C. Beta Actin (Santa Cruz sc-1615 or Cell Signaling #3700) was used as a loading control. Blots were washed, then incubated with HRP-conjugated anti-species secondary (Cell Signaling Technology, Rabbit #7074/ Mouse #7076, 1:1,000) and developed by enhanced chemiluminescence followed by exposure to light-sensitive film or imaging by LI-COR Odyssey Fc. Quantification performed with Image Studio software (LI-COR). MSC CM was analyzed using Ray Biotech Mouse Cytokine Array C-2000 (AAM-CYT-2000) per manufacturer protocols.

**PCR gene expression:**

RNA was extracted with TRIzol (Invitrogen #15596) from F0 and F2 PalII and F0 and F2 DU145 after treatment with MSC CM for 0,1,5,10 and 60 minutes. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4368813). PCR reactions were set up using HotStarTaq Master Mix (Qiagen, #203445). PCR cycle conditions were 95°C for 15 minutes, followed by 35
cycles (95°C for 30 sec, 57°C for IL-28Rα and IL10Rβ or 55°C for IL-28 and 18S; 30 sec, 72°C for 45 seconds. Primers for targets were as follows; Mouse IL-28 Forward 5’-GTCAAGTCTCTGTCCCCAAA -3’, Mouse IL-28 Reverse 5’-GTGGAACCTGCACCTCATGT -3’, Rat IL-28Rα Forward 5’-CCTGTTCTGATGCAAAGCG-3’, Rat IL-28Rα Reverse 5’-AAGTAGGTCACATTGGGGG-3’, Rat IL-10Rβ Forward 5’-GAACGGGAGAGTGGAGCAAA-3’, Rat IL-10Rβ Reverse 5’-ATGCTGAAGCGCCAGTAG-3’, 18S Forward 5’-GTAAACCTGTTGAACCCCATT-3’, 18S Reverse 5’-CCATCCAATCGGTAGTAGCG-3’.

**shRNA and siRNA of IL-28Rα and STAT3:**

Lentivirus expressing human or rat IL-28Rα shRNA (Origene, human; HT144125A-D, Rat; HT144126A-D, scrambled control; TR30033) were generated using standard procedures. PAIII and DU145 cells were seeded into 6-well plates (1x10^6/well) and transduced with retrovirus in 4μg/ml polybrene. After 24 hours the media was replaced with 2mls of 10% serum containing DMEM without antibiotics for 48 hours to allow for recovery. Stable clones were selected via treatment with media containing blasticidin (8μg/ml). Prior to analysis, IL-28Rα levels were analyzed via immunoblot as described. For STAT3 siRNA, 1x10^6 cells from each cell line were seeded into individual 6-well plates and incubated overnight. Cells were transfected using lipofectamine RNAiMAX (Thermo Fisher Scientific # 13778500) with either rat (Origene; SR501698A-C), human (SR321907A-C) or control (SR2004)
siRNAs according to manufacturer’s instructions. At 48 hours post transfection, cells were trypsinized and seeded (3x10^3) in triplicate in 96-well plates for growth analysis at 24, 48 and 72 hours. Remaining cells were lysed and assessed by immunoblot as described for total STAT3.

Analysis of publicly available datasets:

IL-28, IL-28Rα and IL-10Rβ expression levels in control (prostate gland tissue) or prostate adenocarcinoma were performed using the Oncomine platform (www.oncomine.org) [356].

FasL studies:

For PAIII treatment 6x10^4 cells per well were seeded in 48 well plates in triplicate and treated with murine rFasL (R&D Cat # 6128-SA/CF) at varying concentrations. Anti-HA antibody (2.5ug/ml) was used to trimerize and activate the FasL (R&D Cat #MAB060) incubated with or without rFasL at 4C for 1 hour while mixing. Subsequent to 48 hours incubation, cells were lysed, and luminescence was quantified using the Promega luciferase assay. For cleaved caspase-3 immunofluorescence studies, PAIIIs (F0 or F2) were seeded into 8-well chamber slides (LAB-TEK #154534) at 2x10^4 and cultured overnight before treatment with anti-HA alone, 10ng/ml rFasL+HA or 100nM etoposide for 5 hours. Cells were then rinsed with PBS and fixed in 4% PFA at room temperature for 20 minutes. Fixed cells were then blocked for 30 minutes at room temperature in antibody diluting buffer (2% BSA, 0.1% Triton X-100 in PBS). Primary antibodies Cleaved Caspase 3, (Cell Signaling Technology #9661S), 1:400 dilution in antibody diluting buffer; Rabbit IgG Isotype Control, (Thermo Scientific #31235) were incubated
at room temperature for 30 minutes. Aspirate and Cells were then washed 3x in PBS and incubated with secondary antibody (Alexa FluorTM Goat Anti- Rabbit 488, (Invitrogen #A-11034), 1:1,000 dilution in antibody diluting buffer for 30 minutes at room temp in the dark. After washing 3x in PBS, culture chambers were removed and the slides mounted with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, # H-1200). Ten representative images from each group were counted and the number of CC3+ cells were plotted as a percentage to the total number of DAPI stained nuclei.

**QuantSeq analysis:**

Total RNA was extracted using biological replicates collected in triplicate from each, F0 and F2 PalI and F0 and F2 DU145 cell lines using the RNeasy kit (Qiagen Cat# 74104) followed by DNase treatment using the Max Kit (Qiagen Cat # 15200). RNA concentrations were measured using the Qubit RNA BR assay and RNA integrity was assessed using the Agilent 4200 Tapestation. Five hundred nanograms of RNA per sample was processed by the Moffitt Molecular Genomics Core to generate libraries for gene expression analysis using the Lexogen QuantSeq 3’ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen Cat #015) per manufacturer protocols. At least 20 million 101-base single-end sequencing reads per sample were generated on the Illumina NextSeq 500 sequencer. The Bluebee Genomics Platform provided with the QuantSeq kit from Lexogen was used for read for alignment, counting, and differential expression analysis.
**Statistical analysis:**

To determine statistical significance among groups, T-test or analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was performed. A p-value <0.05 was considered as statistically significant. Data are presented as standard error from the mean (S.E.M). All statistical analyses were performed using Graph Pad Prism 6.0 (GraphPad Inc. La Jolla, CA). Further analysis of the data was performed by the Moffitt biostatistics and bioinformatics shared resource cores.
Chapter 6: Conclusions and Clinical Implications:

The data and results from “Chapter 3: Histone Deacetylase Inhibition Prevents the Growth of Primary and Metastatic Osteosarcoma” demonstrates for the first time that osteosarcoma is sensitive both in vitro and in vivo to HDAC inhibition with either the pan HDAC inhibitor panobinostat or the HDAC 1&2 specific inhibitor romidepsin. Panobinostat was shown to be effective in reducing tumor burden in primary osteosarcoma as well as also impairing the growth of established lung metastases and improving overall survival. The preclinical testing also revealed that pre-treatment with panobinostat is successful in inhibiting the lung seeding of osteosarcoma cells.

Since romidepsin inhibited osteosarcoma growth at low nanomolar concentrations similarly to panobinostat, we postulated that HDAC1&2 are essential to osteosarcoma growth. To further elucidate whether one or both of these HDACs are important in supporting the growth of osteosarcoma cells, we used siRNA to knockdown HDAC1 and we observed an increase of HDAC2 levels conversely when we used siRNA to knockdown HDAC2 we observed an upregulation of HDAC1 suggesting that these two HDACs can compensate for each other in osteosarcoma cells. Knockdown of either HDAC1 or 2 alone had no significant impact on osteosarcoma cell growth but knockdown of both simultaneously significantly inhibited growth. When we tested both panobinostat and romidepsin for the treatment of SAOS2LM7, a human lung metastatic cell line, in vivo, we used immunocompromised mice to allow the human cells to engraft
in the lungs. In this model, we observed that while both panobinostat and romidepsin significantly reduced tumor burden in the lungs it was not as efficacious as in the syngeneic models that we previously used. This observation leads us to believe that HDAC inhibition may have a role in stimulating an immune response. Further investigation of the immune infiltrate into the tumors of mice treated with panobinostat and romidepsin is needed to confirm this theory. If this is the case, it is possible that combining immune checkpoint therapies with HDAC inhibition could enhance the efficacy of both treatments. Further investigation into the changes of protein expression using RNA sequencing and proteomic analysis after osteosarcoma cells are treated with HDAC inhibitors will help provide a better understanding of the cellular effects HDAC inhibition has on osteosarcoma cells. This analysis will reveal more specific targets to be evaluated as osteosarcoma treatment opportunities. Overall the results of our experiments provide the needed preclinical data and rationale to help guide the creation of clinical trials using HDAC inhibition for lung metastatic osteosarcoma patients that have failed conventional treatments. Our data also suggests that it may be useful to provide HDAC inhibition to primary osteosarcoma patients without detectable lung metastases as a preventative measure. While our data revealed that HDAC inhibition was effective for suppressing primary osteosarcoma and the resultant lung metastases, HDAC inhibition does not appear to be a viable treatment option for mCRPC patients. A phase 2 trial using romidepsin for mCRPC revealed that there was only minimal antitumor activity with only 2 of 35 patients showing a partial response [357]. Another phase 2 trial testing panobinostat for the treatment of CRCP was determined to be not effective [358]. While HDAC inhibition has yet to be tested specifically for the treatment
of bone metastatic prostate cancer, we took a different approach in search for a therapeutic option for the metastatic skeletal malignancy. To reveal new therapeutic options for bone metastatic prostate cancer patients we examined the cellular and molecular interactions of prostate cancer cells and bone resident mesenchymal stem cells.

The data and results from “Chapter 5: Mesenchymal Stem Cell-Derived Interleukin-28 Can Drive the Selection of Apoptosis Resistant Bone Metastatic Prostate Cancer” reveal that bone-marrow derived MSCs produce IL-28, which triggers rapid apoptosis of bone metastatic prostate cancer cells via IL-28Rα-STAT1 signaling. That has known roles in apoptosis, and in anti-viral and immune responses [323]. However, chronic exposure to MSCs leads to the selection of PCa populations that display a shift to IL-28Rα-STAT3 signaling leading to resistance to IL-28 induced apoptosis, and to conventional chemotherapies such as etoposide and docetaxel. Notably, STAT3 is generally considered to be pro-tumorigenic [324, 328] and is hyperactivated in bone metastatic prostate cancer [325, 359]. Consistent with these findings, treatment of MSC-selected prostate cancer cells with a selective small molecule inhibitor of STAT3, S3I-201, impairs their growth and survival ex vivo and in vivo. Thus, the IL-28Rα-STAT3 signaling circuit represents an attractive and therapeutically tractable vulnerability for bone metastatic prostate cancer. Our findings also indicate that the application of STAT3 inhibitors may re-sensitize prostate cancer cells to chemotherapy and that, given the role of STAT3 activity in the progression of a wide range of cancers [360, 361], the mechanism described herein may have broad applicability to other skeletal malignancies and/or metastases.
If STAT3 inhibition is not effective in treating bone metastatic prostate cancer, there are other factors revealed in the Quant Seq data that represents possible therapeutic targets. The human mitochondrion has a circular double stranded genome that contains 13 protein-coding genes as well as 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) [362]. In our quant seq data from the human prostate cancer cell line DU145, 9 of the 13 mitochondrial protein coding genes were downregulated in the resistant F2 DU145 compared to their F0 parental counterparts (Table 5 and Figure 28). Of the 13 genes highlighted in yellow, 12 are part of the 4 complexes that make up the electron transport chain (ETC). The downregulation of these genes could possibly be explained by the fact that resistant cells are undergoing a metabolomic shift toward glycolysis as explained by the Warburg effect [5]. Suppression of mitochondrial gene expression was observed in multiple different tumor types when compared to normal matched tissue [363]. The downregulation of the mitochondrial genes could be mediated by MSC induced activation of mitochondrial STAT3. Mitochondrial STAT3 enhances that activity of both complex 1 and 2 of the ETC [352, 364], which could be the reason that mitochondrial genes are downregulated in MSC-educated prostate cancer cells. The nuclear function of STAT3 as a transcription factor has been well studied but the roles of STAT3 in the mitochondria are still being discovered. While the tyrosine phosphorylation of STAT3 at tyrosine 705 is thought to be primarily responsible for the dimerization and activation as a nuclear transcription factor [365]. STAT3 can also be phosphorylated at serine 727 by mitogen activated protein kinases (MAPK) [366]. This serine phosphorylation can cause STAT3 interaction with GRIM-19 a chaperone that shuttles STAT3 to the mitochondria [367, 368]. There is increasing
evidence that mitochondrial STAT3 is responsible for cancer progression as well as resistance to apoptosis. Mitochondrial STAT3 binding mitochondrial DNA and regulating gene expression was implicated in tumorigenesis of keratinocytes [369]. Mitochondrial STAT3 has been shown to reduce radical oxygen species (ROS) increasing the growth in breast cancer cells [370]. Further research is warranted to study targeting mitochondrial STAT3 to increase therapeutic benefit of other therapies currently being used for bone metastatic CRPC.

**Table 5:** Top 20 downregulated genes in DU145 cells after treatment with MSC CM. Mitochondrial Genes highlighted in yellow

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene</th>
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<tbody>
<tr>
<td>1</td>
<td>IL24</td>
</tr>
<tr>
<td>2</td>
<td>MYADML2</td>
</tr>
<tr>
<td>3</td>
<td>MMP1</td>
</tr>
<tr>
<td>4</td>
<td>MTATP6P1</td>
</tr>
<tr>
<td>5</td>
<td>MT-ATP6</td>
</tr>
<tr>
<td>6</td>
<td>LOX</td>
</tr>
<tr>
<td>7</td>
<td>KRTAP2-3</td>
</tr>
<tr>
<td>8</td>
<td>MPZL2</td>
</tr>
<tr>
<td>9</td>
<td>SPOCD1</td>
</tr>
<tr>
<td>10</td>
<td>MT-ND1</td>
</tr>
<tr>
<td>11</td>
<td>MT-CYB</td>
</tr>
<tr>
<td>12</td>
<td>MT-TQ</td>
</tr>
<tr>
<td>13</td>
<td>EVA1A</td>
</tr>
<tr>
<td>14</td>
<td>RPL22L1</td>
</tr>
<tr>
<td>15</td>
<td>MT-ND4</td>
</tr>
<tr>
<td>16</td>
<td>MT-ND2</td>
</tr>
<tr>
<td>17</td>
<td>MT-CO3</td>
</tr>
<tr>
<td>18</td>
<td>ZNF185</td>
</tr>
<tr>
<td>19</td>
<td>MT-ND5</td>
</tr>
<tr>
<td>20</td>
<td>MMP14</td>
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Currently, the only approved therapy that has improved overall survival for bone metastatic CRPC is radium 223 (also known as Xofigo) [371]. There are currently 38 clinical trials for bone metastatic prostate cancer that are either recruiting or active and not recruiting. Of the 38 trials 16 of them include radium 223 [146]. A recent trial tested abiraterone with or without radium 223 in bone metastatic prostate cancer patients. Abiraterone alone had shown increased overall survival for CRPC patients so
combination with radium 223 was expected to increase overall survival and decrease in skeletal related events (SREs) for bone metastatic patients. Unfortunately, for unknown reasons the trial revealed that addition of radium 223 to abiraterone did not significantly increase in skeletal event free survival [372]. We hope the results from our studies that revealed the targeting STAT3 with a small molecule inhibitor effective in treating apoptosis resistant prostate cancer cells in combination with docetaxel, abiraterone and or radium 223 might extend overall survival and move us closer to curing bone metastatic prostate cancer. Further research is warranted in testing how these combinations would be best administered.

Adaptive therapy is a concept that incorporates Darwinian evolution and population ecology principals. Tumors are heterogeneous and contain both sensitive and resistant cell populations. There are costs for cancer cells that harbor mechanisms of resistance. In many cases, sensitive cells outgrow resistant populations which make up a small percentage of the entire tumor. When treatments are administered at the maximum tolerated dose, they can significantly reduce tumor volume, but this eradication of the sensitive cells removes the competition for resources in the tumor microenvironment and allows the resistant cells to reestablish a majority of the tumor outgrowth [373-375]. This new paradigm in cancer treatment has been effectively implemented in treating metastatic CRPC. Using a patient specific-dosing schedule of abiraterone, based on recurring PSA levels, a recent study approximately doubled the median time to progression from 16.5 months to 27 months while administering half the standard dosage. [376]. Using the same concept of adaptive therapy has recently been suggested for use in pediatric sarcomas as well [377].
Overall, the new data collected in our studies of both primary and metastatic skeletal malignancies in combination with adaptive therapy will help create new treatment strategies that will move us closer to a cure for these patients. For example, if we combined strategies such as STAT3 inhibition in combination with ADT and chemotherapies, using patient specific adaptive treatment strategies we may be able to increase overall survival, decrease SREs and move one step closer to curing bone metastatic prostate cancer. Similarly, targeting epigenetic vulnerabilities with either HDAC inhibitors or targeted therapies revealed by HDAC inhibition we can hopefully increase overall survival for lung metastatic osteosarcoma patients. If administered in combination with conventional chemotherapies, developing unique treatment strategies for each patient we hopefully will discover novel treatment strategies that will cure patients with lung metastatic osteosarcoma.
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[cited 2019 April 1]; Available from: [https://www.oncomine.org/](https://www.oncomine.org/).


Appendices:
Appendix A: Copyright Permissions from Elsevier Publications

I sought permission to use portions of my first author publication “Re-calculting! Navigating through the osteosarcoma treatment roadblock” that was published in Pharmacological Research [110], I was directed to the copyright permissions section of the Elsevier publications website where it is stated that authors can include their articles in full or in part in a thesis or dissertation. See screen shot below.

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Do I need to request permission to text mine Elsevier content? +

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I sought permission to use my recent first author publication titled "Histone deacetylase inhibition prevents the growth of primary and metastatic osteosarcoma" published in International Journal of Cancer, I was directed to the copyright and permissions section for Wiley publications. It is stated that as a Wiley author you can include your article in your thesis. See screenshot below.

The manuscript was accepted for publication on April 12th 2020 and has a manuscript ID: IJC-20-0101.R1 but the publication process has not been completed yet as of June 24th 2020 so I do not have an official reference for the manuscript.