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TUNGSTEN-ENHANCED BREAST CANCER METASTASIS TO THE BONE IS ASSOCIATED WITH ALTERATIONS IN THE BONE NICHE.

Cameron J. Chock
University of New Mexico

Alicia Bolt
University of New Mexico

Cameron J Chock

Candidate

College of Pharmacy - Pharmaceutical Sciences

Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

Dr. Alicia Bolt, Chairperson

Dr. Helen Hathaway

Dr. Matthew Campen

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by

CAMERON J CHOCK, BS

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Tungsten-enhanced breast cancer metastasis to the bone is associated with alterations in the bone niche.

by Cameron J Chock

B.S., Biology, University of New Mexico, 2017

Pharm.D., College of Pharmacy, University of New Mexico, 2022

M.S., Pharmaceutical Sciences, University of New Mexico, 2022

ABSTRACT

The objective of this thesis project was to investigate the effects of tungsten on breast cancer metastasis to the bone niche, using the 4T1 orthotopic breast cancer model. Oral tungsten (15 ppm) exposure did not affect primary tumor growth. However, following tungsten exposure there were marked changes in the bone niche, including increased metastasis of 4T1 tumor cells and increased osteolysis. Further analysis of the bone niche indicates that enhanced metastasis is associated with a pro-tumorigenic immune suppressive environment, including increased number of granulocytic myeloid derived suppressor cells and increased gene expression of the pro-inflammatory cytokine IL-1β and the activated fibroblast marker α smooth muscle actin. These results suggest that tungsten accumulation in the bone is changing the bone niche to create a pro-tumor immune suppressive environment. The exact mechanisms behind this are multifactorial, but evidence suggests tungsten may be affecting breast cancer cells to enhance homing and colonization within the bone niche.

Table of Contents

List of Figures

Chapter 1 Introduction

Tungsten: Background and an Environmental Toxicant

Tungsten is a rare transition metal with the atomic number 74. It has many uses and applications within industrial, military, and medical sectors including armor penetrating munitions, explosive devises, jewelry, power tools, welding electrodes, X-ray equipment, electronics, golf clubs, and implanted medical devices. (EPA, 2017) This is due to several desirable properties including strength, flexibility, and good conductive properties, in addition to having one of the highest melting and boiling points of all metals. (Keith et al., 2007) Tungsten was also favored for its assumed biological inertness when used within the body in the case of coils used for embolisms and shields in breast cancer patients. However, this is rapidly changing as more information is brought to light concerning the toxicity profile of tungsten. While overall exposure to tungsten in the general US population is low, current data indicates that particular subsets of the population are at increased risk of exposure to high levels of tungsten including those in occupational, environmental, medical and military settings. (EPA, 2017; Keith et al., 2015; NTP, 2002)

Individuals all over the world can be exposed to high levels of tungsten in many diverse settings, due to its use in many different types of industrial applications. Occupational exposure often occurs through mining and industrial

processing of tungsten metal and tungsten-cobalt or tungsten carbide-cobalt alloys for use in many different applications. Workers are exposed through points of contact such as dermal and inhalation exposure. The National Institute for Occupational Safety and Health (NIOSH) and Occupational Safety and Health Administration (OSHA) have regulatory limits of a time-weighted average concentration for up to a 10-hour workday, during a 40-hour workweek, of 5 mg/m^3 and a short-term exposure limit for 15 minutes at 10 mg/m^3 of insoluble tungsten compound inhalation. However, occupational workers are exposed to aerosolized tungsten particles. Data from the early 2000s reported ambient air tungsten concentrations ranging between 3.3 to 417 $\mu g/m^3$ in hard metal manufacturing plants. However, concentrations as high as 6.1 mg/m^3 were reported in the 1970s. This resulted in urinary tungsten concentrations ranging from 0.33 to 168.6 µg/g creatinine (ppb), with concentrations as high as 1.1 ppm reported back in the 70s. The highest average urinary tungsten concentrations were found in heavy metal plant workers that manufacture and grind heavy metal alloys, including tungsten carbide, who had reported ranges of 10.6 – 168.6 µg of tungsten /g creatinine in their urine. (Kraus et al., 2001; "Occupational exposure to tungsten and cemented tungsten carbide," 1977)

In the environment, tungsten exists naturally in the form of minerals including wolframite ($[FeMn]WO_4$) and scheelite ($CaWO_4$), which both break down to tungstates. As we utilize tungsten in industrial applications tungsten changes forms into tungsten metal and tungsten carbide. However, tungstates

(WO_4^{2-}) are the most soluble and comprise the most bioavailable forms of tungsten and can contaminate drinking water sources. (Clausen & Korte, 2009; Mamuro et al., 1971; Keith et al., 2007; EPA 2017) In addition, tungsten and tungsten carbide metal alloys can enter into the environment through waste produced from mining operations and industrial sites processing tungsten. Human exposure can occur through aerosolized particles as well as consumption of contaminated water that most often occurs in regions previously home to tungsten mining and other sites related to the production of tungsten metals and alloys. (EPA, 2017) The Environmental Protection Agency (EPA) has no regulatory standards set for tungsten in drinking water or air, and tungsten is not routinely monitored in the environment. However, three states have enacted safety limits with Indiana being the only state that has soil (88 ppm) and groundwater (0.016 ppm) screening levels. (EPA, 2017) The state of North Carolina has preliminary protection of groundwater goals for tungsten (8.4 ppm; EPA, 2017), and Texas has soil (820 ppm) and groundwater (7.3 ppm) protective concentration levels for sodium tungstate dihydride. (EPA, 2017) Evidence of elevated levels of tungsten in the Southwestern US makes this information even more shocking due to possible health related risks. Recent data, from Drs. Debra MacKenzie and Johnnye Lewis' group at the University of New Mexico College of Pharmacy have shown that 44.3% of women from the Navajo Nation have urinary tungsten levels above the 75th percentile of concentrations found in the general US population (NHANES Database, Data Unpublished). In addition, the levels of tungsten in the drinking water near the town of Fallon, NV have been

measured as high as 742 ppb (Seiler et al 2005), resulting in urinary tungsten concentrations as high as 1.2 ppb (reference values = 0.08 ppb). Importantly, these high tungsten concentrations in Fallon, NV were associated with an increased incidence of pediatric leukemia cases in children living in this area, but no correlative link was established. (Rubin et al., 2007; Schell & Pardus, 2008)

Tungsten is used in multiple implanted medical devices due to its assumed biological inertness within the body. However, this use of tungsten-containing devices can cause erosion in the body leading to increased concentrations of urinary tungsten within patients. In a recent study a cohort of breast cancer patients was accidentally exposed to tungsten following intraoperative radiotherapy treatment using a tungsten-based shield. (Bolt et al., 2015) The levels of tungsten in the urine of some of these patient's, even 2.5 years following the operation, were 4x higher than concentrations reported in the general US population. Tungsten coils are also utilized during routine peripheral vascular embolization catheter-based procedures meant for precise occlusion of abnormal blood flow in a blood vessel. These coils have been reported to corrode and degrade over time and increase urinary tungsten concentrations which have been reported to range between 1.98 and 837.7 ppb. (Bachthaler et al., 2004; Barrett et al., 2000)

Military exposure to tungsten occurs following its rapid replacement of lead and uranium in many different types of munitions including armor

penetrating bullets and improvised explosive devices. Therefore, veterans could be chronically exposed to tungsten through explosively aerosolized products or imbedded shrapnel, which could cause a slow release of tungsten within the body over time. After surveying a cohort of US veterans with embedded shrapnel wounds it was found that 11.6 % of the veterans had tungsten concentrations higher than the NHANES database upper limit reference control value of 2.70 µg/g creatinine. (Gaitens et al., 2016) In addition, tungsten was the second most common metal found elevated in the urine in this cohort.

Tungsten and the Bone

In adults it is normal for there to be remodeling of the bone matrix, as bone is turned over in a balanced process where it is broken down and built back up primarily through two different cell types. The bone undergoes destruction, also called resorption, which is carried out by osteoclasts, and undergoes formation by cells called osteoblasts. There are many different factors that regulate these two processes and maintain the homeostasis of the bone microenvironment. However, emerging data suggests that tungsten may play a role in disrupting the bone microenvironment and, therefore, could be promoting breast cancer metastasis to the bone through this mechanism. (Bolt et al., 2016; Chou et al., 2021)

Importantly, Dr. Bolt has shown that tungsten accumulates in the bone and the bone is a site of long-term storage and toxicity. (Bolt et al., 2016; Bolt et al., 2015) It has been shown that tungsten skews bone marrow mesenchymal stromal cell (MSC) differentiation by inhibiting osteoblastogenesis and promoting adipogenesis. *In vitro* studies indicated that tungsten augmented rosiglitazone-mediated adipogenesis (Bolt et al., 2016), suggesting potential enhancement of the peroxisome proliferator activated receptor gamma (PPARγ) pathway because rosiglitazone is a PPARγ agonist. (Zhang et al., 2015) *In vivo*, it was also demonstrated that tungsten can enhance adipogenesis in the bone. However, these results were shown to be age- and sex-dependent, as only young (6-8 weeks) male mice treated with tungsten showed an enhancement of adipogenesis. However, when aged (9 months) mice were exposed to tungsten, only the females showed an enhancement of adipogenesis. (Bolt et al., 2016) Therefore, it is suggested that multiple mechanisms besides the PPARγ mediated pathway are affected by chronic tungsten exposure within the bone. There is also evidence that tungsten can affect osteoclasts. In one paper from the research group of Dr. Koren Mann, they observed significantly elevated osteoclast numbers in the trabecular bone of femurs following oral exposure to tungsten in male, but not female mice. (Chou et al., 2021) Furthermore, they observed that tungsten enhanced receptor activator of nuclear factor kappa-B ligand (RANKL)-induced differentiation into tartrate-resistant acid phosphatase (TRAP)-positive mononucleated osteoclasts through enhanced increased gene expression of differentiated osteoclast markers Nfatc1, Acp5, and Ctsk. This

suggests that tungsten enhances sex-specific osteoclast differentiation and implicates tungsten as a modulator of bone homeostasis. Therefore, tungsten may have a direct action on the osteoclast action and prolific disruption of the bone microenvironment, which could influence breast cancer metastasis to the bone.

Interestingly, disease state also influences tungsten concentration within the bone. Breast tumor-bearing mice exposed to tungsten had higher tungsten concentrations when compared with non-tumor bearing mice. (Bolt et al., 2015) Preliminary data from the Bolt lab indicates an increase in osteoclast promoting cytokine secretion of macrophage colony stimulating factor (MSCF) and interleukin 15 (IL-15) from bone marrow stromal cells cultured with 4T1 breast cancer cell conditioned media and tungsten *in vitro* (Data Unpublished).

Tungsten and Carcinogenesis

There is significant evidence to support that tungsten-containing compounds are genotoxic and contribute to a pro-tumorigenic process. There is some epidemiological data providing evidence that exposure to tungsten contributes to the carcinogenesis process. The previously mention levels of tungsten in the drinking water near the town of Fallon, NV, resulting in urinary tungsten concentrations as high as 1.2 ppb (reference values = 0.08 ppb) are of great concern. Importantly, these high tungsten concentrations in Fallon, NV

were associated with an increased incidence of pediatric leukemia cases in children living in this area, but no correlative link was established. (Rubin et al., 2007; Schell & Pardus, 2008) Furthermore, workers exposed to tungsten carbide-cobalt dust in the hard metal manufacturing industry have a higher incidence of mortality from lung cancer after adjusting for smoking status; however, the contribution of tungsten versus cobalt on the carcinogenic effects remains to be defined. (Moulin et al., 1998; Wild et al., 2000)

In vitro, small particles of metallic tungsten have been shown to induce single strand breaks in supercoiled plasmid DNA (Mazus et al., 2000), and have been shown in bacterial mutagenic assays to induce mutagenic activity. (Sora et al., 1986; Ulitzur & Barak, 1988) In another study, tungsten carbide alone caused minimal DNA damage, suggesting that a synergistic effect between tungsten carbide and cobalt induced DNA damage. Furthermore, a co-treatment with a hydroxyl radical scavenger reduced DNA strand breaks, reliably suggesting that DNA damage is occurring in the presence of ROS generation. (Anard D et al., 1997)

Evidence also exists suggesting that tungsten enhances cell proliferation and tumorigenesis *in vivo*, such as a study where rats implanted with tungsten-based pellets in a model meant to replicate shrapnel injuries. These rats developed highly aggressive forms of rhabdomyosarcoma. (Emond et al., 2015a, 2015b; Kalinich et al., 2005) However, oral sodium tungstate did not alter the

initiation of lung cancer or the incidence of mammary gland tumors following benzo[a]pyrene exposure (Gunnison et al., 1988). This suggests that the effect of tungsten to promote cancer is not consistent in all carcinogen-induced models, and therefore exposure to oral tungstates may result in drastically different results from implanted tungsten metal alloys.

Investigation of the cohort of breast cancer patients accidently exposed to tungsten led Dr. Bolt to investigate the role of tungsten on breast cancer progression and metastasis. Interestingly, tungsten enhanced lung metastasis in an orthotopic breast cancer mouse model. The increased metastasis was not due to an increase in the primary tumor size, but instead correlated with changes in the tumor microenvironment known to promote metastasis (Bolt et al., 2015). Thus, one explanation for the conflicting results regarding tungsten as a tumor promoter may be specific to the type of tumor and/or the specific tumor microenvironment components involved. This increase in breast cancer lung metastasis following tungsten exposure was associated with an increased number of cancer-associated fibroblasts, myeloid-derived suppressor cells (MDSCs), and matrix metalloproteases in the surrounding microenvironment. This work was novel because it identified, for the first time, that tungsten exposure can drive tumor promotion by targeting the tumor microenvironment.

Breast cancer: A Brief Overview

Breast cancer is a type of cancer that starts in the breast tissue. Most types of breast cancer start as a mutation in the DNA particularly in either the cells of the ducts that carry milk to the nipple or in the lobules, which are the glands that make breast milk. Which cells are affected also determines how breast cancers are classified. One such type of cancer is called a carcinoma, which is a tumor that forms in the epithelial cells that line organs and tissues throughout the body. Most breast cancers are usually a more specific type of carcinoma that specifically forms in the glands of the body that secrete mucus and other fluids called an adenocarcinoma. (*Breast Cancer Treatment (Adult) (PDQ®)–Patient Version*; Wellings & Jensen, 1973)

Once breast cancer is identified, it is further classified according to invasiveness. While breast cancer forms within certain structures in the breast it can start to spread into other tissues within the breast. The term invasive applies to any form of breast cancer that has spread into the surrounding breast tissue. There are multiple terms used to classify how invasive breast cancers are, but there are a few common clinical terms used to describe to patients. One such term is ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), which are classified as non-invasive or as pre-invasive breast cancers. If breast cancer is invasive the most common types of classifications are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), which refer to cancers

that start in the ductal and lobular tissue respectively. Invasive ductal carcinoma is the most common of all breast cancers and contributes to approximately 70-80% of new diagnoses. (*Breast Cancer Treatment (Adult) (PDQ®)–Patient Version*; Wellings & Jensen, 1973)

The spread of breast cancer is initially limited to the breast tissue, but over time it can also start to spread to other parts of the body. In order to better classify the degree to which cancer has spread, medical professionals use a method called staging. There are three different criteria upon which stage is based, and those are the size of the tumor, has the tumor spread to the lymph nodes, and if the tumor has metastasized or spread to a more distant part of the body. Stages of cancer are on a scale of 0-4, where 0 deals mostly with DCIS and LCIS, 1-3 are still localized to the breast tissue and regional lymph nodes, and four is for metastatic cancer which has spread to distant parts of the body. (Bleyer & Welch, 2012; Gradishar et al., 2021)

Once breast cancer is identified, treatment follows; however, treatment of breast cancer is a complex process that relies on many factors such as progression and other genetic factors. One such factor that is often considered is the expression of cell surface receptors that can be targeted for treatment. Of these receptors, three are often better targets than the rest. Two receptors target the female hormones estrogen and progesterone, while the third is for a protein called human epidermal growth factor (HER2). When breast cancer is

discovered, it is not uncommon to identify which of these receptors are sensitive to develop a treatment plan where positive receptors respond to directed treatment and negative ones do not. However, if a cancer is negative to all three receptors it is called “triple negative” breast cancer. This form of cancer is difficult to treat due to its lack of treatment targets, which may also lead to an increased likelihood of relapse compared to other types of breast cancer. Furthermore, it is also considered aggressive due to its fast growth and therefore is more likely to have spread by the time of discovery. Due to all of these factors there is a more negative outlook for triple negative breast cancer when compared to other types of breast cancer. (Bleyer & Welch, 2012; Foulkes et al., 2010)

Importantly, cancer formation is not readily apparent as signs and symptoms take time to develop. One of the most common early signs and symptoms of breast cancer often manifest themselves as a new lump or mass within the breast tissue. As the disease progresses more symptoms may arise in the form of constant pain, changes to skin texture and color, and discharge from the nipple, to name a few. If cancer has reached a state to where it has metastasized to other sites in the body, many other very unpleasant side effects may also be experienced. Common sites of metastasis include the bone, lung, liver, and brain. Metastasis to these sites often results in bone and joint pain, chest pain, difficulty breathing and persistent cough, jaundice, and neurological symptoms. (Akhtari et al., 2008; Gradishar et al., 2021)

It is estimated that 1 in 8 US women will develop invasive breast cancer over their lifetime, with an expected 287,850 new breast cancer cases and 43,250 deaths to occur in the US in 2022. (*Cancer Statistics*) Breast cancer has the second highest mortality rate for cancers in women, with only lung cancer surpassing it. Since 2007 breast cancer death rates have remained steady in women younger than 50, but have decreased in older women. The decreased rate of mortality is believed to be due to earlier screening and increased awareness, as well as better treatments. Many women with breast cancer are often given a relative survival rate, which works by comparing the survival rate of women with the same type and stage of breast cancer to the survival rate of overall population. Most commonly, a five-year relative survival rate is used where they compare the average rate of survival for a woman with breast cancer in comparison with other women over the same time period. This means that if the five-year relative survival rate for a stage two cancer is 75% it indicates that women with that type of cancer are about 75% as likely as women who do not have cancer to live for five years after being diagnosed. (*Breast Cancer Facts and Statistics*; *Cancer Statistics*)

Breast Cancer Metastasis to the Bone and the Bone Microenvironment

While the mortality associated with breast cancer has many causes, one factor is invasiveness, with metastatic breast cancer having a poor prognosis. (*Breast Cancer Facts and Statistics*; *Cancer Statistics*) Therefore, it is important

to identify factors that can influence metastasis. Breast cancer can progress to other sites in the body besides the lungs, one such site is the bone. It has been estimated that 70% of breast cancer patients with advanced cancer have bone metastases. (Akhtari et al., 2008) Bone metastases are incurable and have limited therapeutic options, making them very difficult to treat and devastating for the patient. Such treatments require extensive identification of the diagnostic markers in triple negative breast cancer, which is a key focus of our research. Treatments include a composite of programmed death – ligand 1 (PD-L1) inhibitors, which block the interaction of PD-L1 with programmed cell death protein 1 (PD-1; Syn et al., 2017; Wei et al., 1987) or use of anthracyclines which intercalate with DNA and interfere with DNA metabolism and RNA production through the inhibition of topoisomerase II. (Shen et al., 2017) Therapy aimed at specifically controlling symptomatic bone metastases is administered through the use of radiotherapy to decrease local symptoms and bisphosphonates to reduce skeletal complications (i.e. fractures) and risk of hypercalcemia. (Gennari et al., 2021) A better understanding of the molecular mechanisms that regulate how breast cancer metastasizes to specific metastatic niches, in particular the bone, will help to develop effective therapeutic interventions. (Gradishar et al., 2021)

Bone metastasis is a complex process of which many factors are involved. One potential mechanism, termed the “seed and soil” theory of metastasis, suggests that the microenvironment of the pre-metastatic niche dictates where and to what extent metastasis occurs. It is believed that this microenvironment is

primed by invading tumor cells, which serve to further enrich the pre-metastatic niche. (Le Pape et al., 2016; Mercer et al., 2004)

There are multiple factors released by breast cancer cells that enhance tumor cell homing to the bone niche and drive metastasis (Figure 1). Factors derived from breast cancer tumor cells include osteopontin (OPN; Anborgh et al., 2010; McAllister et al., 2008), a promoter in both bone marrow cell migration and tumor cell proliferation; heparanase (HPSE; Kelly et al., 2005), which increases bone reabsorption by reducing heparin sulfate chain length in the extracellular matrix; and parathyroid hormone-related protein (PTHrP; Guise et al., 1996), another factor used to promote bone resorption.

However, priming of the niche is only one part of the “seed and soil” theory of metastasis. Once breast cancer cells are established within the bone, they begin to adapt the microenvironment in the bone niche to make it more hospitable. In the bone, some of the released factors are PTHrP, which is readily secreted by breast cancer cells which continues to promote bone resorption, and prostaglandin-endoperoxide synthase 2 (COX-2), which has a known role in supporting the development and progression of bone metastases by controlling prostaglandin mediated bone resorption. (Singh et al., 2007; Yin et al., 1999) The release of osteolytic factors such as interleukin 11 (IL-11), continue to encourage maturation of osteoclast's furthering bone osteolysis. (Zhang et al., 1998) Increases in matrix metalloproteinase 1 (MMP1) and IL-11 (Lu et al., 2009) are

also shown to be increased by tumor cell presence. This altered balance combined with tumor cell ability to stimulate bone resorption results in an increase of osteoblast production of RANKL (Lu et al., 2009). Interleukin 1 beta (IL-1β), a pro-inflammatory cytokine that signals homing to the bone, has also been shown as a possible factor of concern (Laulicht et al., 2015). Another factor hypoxia-inducible growth factor-1 (Hif-1a) inhibits osteoblast differentiation and promotes osteoclastogenesis, which supports osteolysis by increasing bone resorption and allows for further tumor growth. (Sterling et al., 2006) Finally, an increase in connective tissue growth factor (CTGF) in the bone triggers osteoblast proliferation, which further activates osteoclasts leading to increased osteolysis within the bone (Figure 1). (Shupp et al., 2018; Weilbaecher et al., 2011)

It has been established that breast cancer cells may also continue to express factors that increase recruitment or homing to the bone microenvironment. Factors that contribute to this recruitment that are expressed in this microenvironment include avb3 integrin ($\alpha_V\beta_3$), which promotes adhesion of breast cancer cells in the bone, is closely associated with bone metastasis and increased activity of the receptor of RANKL (RANK; Jones et al., 2006) as it also mediates osteoclast-induced bone reabsorption therefore supporting tumor colonization of the bone. Increased expression of CXC receptor 4 (CXCR4; Liang et al., 2004) on the surface of tumor cells is also seen, which has a role in the binding to the chemokine CXC ligand 12 (CXCL12) on bone marrow stromal

cells. (Liang et al., 2004) CXCL12 is a potent chemoattractant of hematopoietic stem cells (HSCs; Liang et al., 2004) that can use many of the same physiological mechanisms to attract tumor cells when homing to the bone. This interaction may further upregulate avb3 integrin levels, which will only serve to further enhance adhesion of breast cancer cells in the bone microenvironment.

While the previously mentioned factors were secreted directly by tumor cells it is also suggested that bone cells such as osteoclasts can express transcription factors to further support breast cancer growth within the bone (Figure 1). Interestingly some factors secreted by cancerous cells play a different, but pro-tumorgenic, role when also secreted by osteoclasts. For example, transforming growth factor beta (TGF-B) allows cancerous cells to bind more successfully to the extracellular matrix thus evading the action of some cancer drugs. (Lu et al., 2009) However, some factors are unique to the bone microenvironment such as runt-related transcription factor 2 (RUNX2), which regulates matrix metallopeptidase 9 (MMP9) transcription, which leads to the break down the extracellular matrix increasing tumor cell invasion. (Pratap et al., 2005) However, what effect these alterations in the bone microenvironment, in the presence of tungsten, have on the potential to drive breast cancer to metastasize to the bone are unknown.

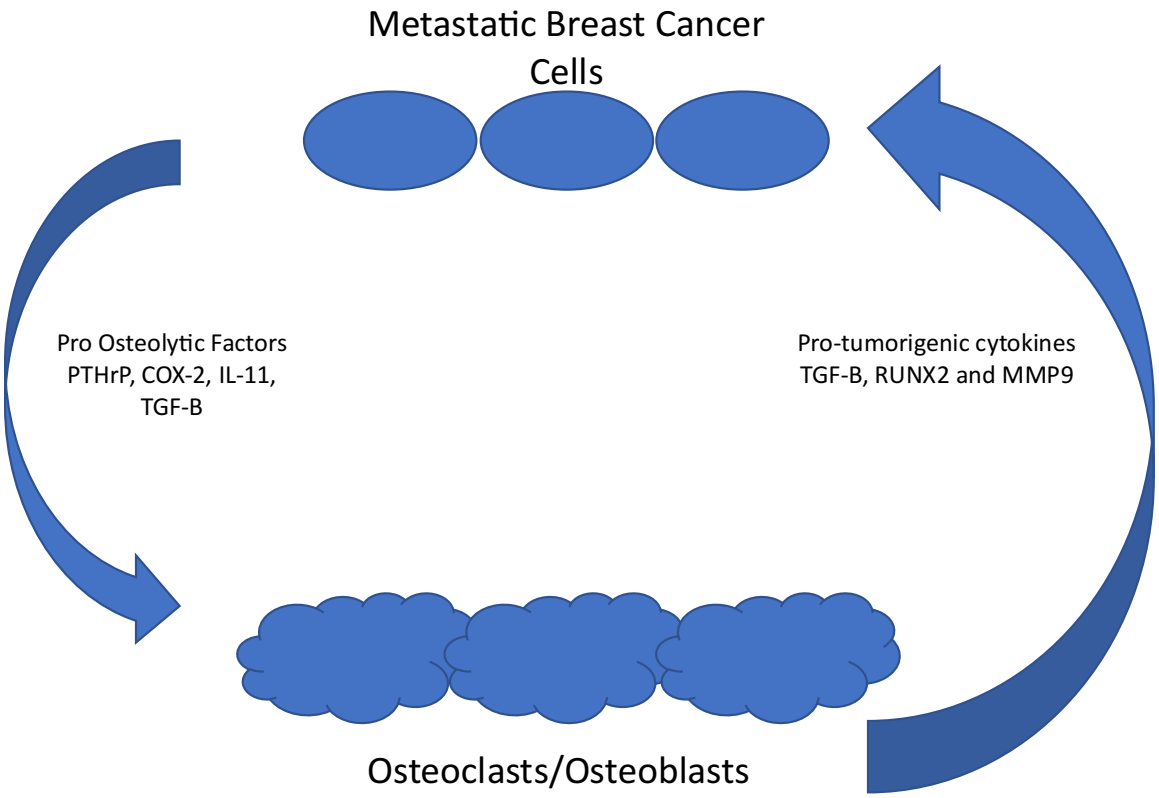

Figure 1. The bone pre-metastatic niche contains multiple factors that can be altered by the presence of metastatic breast cancer cells. *These include pre osteolytic factors such as: PTHrP, COX-2, IL-11, and TGF-B. Pro-Tumorigenic cytokines also play a large role in recruitment and proliferation such as: TGF-B, RUNX2 and MMP9.*

Furthermore, MDSC, a heterogeneous population of immature cells with immunosuppressive ability also are affected by tumor cells. (Ostrand-Rosenberg, 2021) Many biological entities such as MDSCs are immune suppressive and play a role in tumor maintenance and progression. MDSCs also obstruct therapies that seek to treat cancer through both immunotherapy and other non-immune means. (Ostrand-Rosenberg, 2021) Another source of concern are activated myofibroblasts which are the predominant source of production of collagens needed to form the scar and express contractile proteins which includes alpha smooth muscle actin (αSMA). In a carcinogenisis, which can act as a non-healing wound, myofibroblasts can be deficient or fulfill other functions such as the production of proinvasive proteinases. (Basset et al., 1990)

Study Question, Specific Aims and Hypotheses

Overarching Hypothesis:

Tungsten will enhance breast cancer metastasis to the bone by altering bone remodeling through increased osteoclast activity within the bone microenvironment.

Specific Aims:

Aim 1: Assess breast cancer metastasis to the bone following oral exposure to tungsten in vivo.

> *Hypothesis for Aim 1:* Tungsten will enhance breast cancer metastasis to the bone niche.

Aim 2: Evaluate makers of bone remodeling in the bone of tumor-bearing mice following tungsten exposure *in vivo*.

> *Hypothesis for Aim 2:* Tungsten will increase the number of osteoclasts in the bone metastatic niche and increase pro-osteolytic cytokines and signals in the bone niche

Chapter 2: Tungsten Exposure Enhances Bone Metastasis and Osteolysis in 4T1 Breast Cancer Mice

Introduction

It is estimated that 1 in 8 US women will develop invasive breast cancer over their lifetime. With an expected 287,850 new breast cancer cases and 43,250 deaths to occur in the US in 2022. (*Cancer Statistics*) Breast cancer has the second highest mortality rate for cancers in women, with only lung cancer surpassing it. While the mortality associated with breast cancer has many causes, one factor is invasiveness with metastatic breast cancer having a poor prognosis. (*Cancer Statistics*) Therefore, it is important to identify factors that can influence metastasis.

Tungsten is an emerging toxicant due to increased human exposure, yet limited of knowledge of human health risks. One large research gap is our knowledge of the potential carcinogenic/tumorigenic effects of tungsten exposure, despite some compelling epidemiological data. Elevated levels of tungsten in the drinking water near the town of Fallon, NV resulting in urinary tungsten concentrations as high as 1.2 ppb (reference values = 0.08 ppb) are of great concern. Importantly, these high tungsten concentrations in Fallon, NV were associated with an increased incidence of pediatric leukemia cases in children living in this area, however a correlative link was not established. (Rubin

et al., 2007; Schell & Pardus, 2008) Furthermore, workers exposed to tungsten carbide-cobalt dust in the hard metal manufacturing industry have a higher incidence of mortality from lung cancer after adjusting for smoking status; however, the contribution of tungsten versus cobalt on the carcinogenic effects remains to be defined. (Moulin et al., 1998; Wild et al., 2000)

Importantly, there have been multiple animal studies that provide evidence regarding tungsten's role in enhancing tumor progression. For example, rats exposed to sodium tungstate in drinking water had significantly more mammary tumors than rats treated with mutagen alone. (Wei et al., 1987) Rats implanted with tungsten based (W/Ni/Co) pellets to model embedded shrapnel injuries developed highly aggressive forms of rhabdomyosarcoma. (Kalinich et al., 2005) This study was replicated in a follow-up study in B6C3F1 mice.(Emond et al., 2015a) Mice implanted with the W/Ni/Co pellets developed rhabdomyosarcoma tumors around the pellet, but in contrast to the rat model, the mice did not develop aggressive, metastatic tumors. (Emond et al., 2015a) Furthermore, the individual contribution of each metal to the tumorigenesis process was assessed. Interestingly, only mice implanted with either tungsten, cobalt, or tungsten/cobalt pellets developed malignant sarcomas, but significantly less than the 80%tumor incidence was found in W/Ni/Co-implanted mice. (Emond et al., 2015b) These data suggest that the synergistic effects of all three metals in combination drive tumorigenesis to a greater extent than any
of the metals alone.

In a recent study, a cohort of breast cancer patients was accidently exposed to tungsten following intraoperative radiotherapy treatment using a tungsten-based shield. Tungsten could be detected in the circulation for years after exposure, which indicates that tungsten did not remain confined to the breast tissue causing chronic exposure to elevated levels of tungsten (Bolt et al., 2015). This incident led our lab to investigate the role of tungsten on breast cancer progression and metastasis. They found that tungsten exposure increased breast cancer progression by enhancing metastasis to the lungs, using an aggressive mouse mammary cancer model (Bolt et al., 2015). This increase in lung metastasis was associated with an increased number of cancer-associated fibroblasts, myeloid-derived suppressor cells, and matrix-metalloproteases in the surrounding microenvironment. This work was novel because it identified, for the first time, that tungsten exposure can drive tumor promotion by targeting the tumor microenvironment

Breast cancer can progress to other sites in the body besides the lungs, one such site is the bone. It has been estimated that 70% of breast cancer patients with advanced cancer have bone metastases. (Akhtari et al., 2008) Bone metastases are incurable and have limited therapeutic options, making them very difficult to treat and devastating for the patient. Better understanding of the molecular mechanisms that regulate how breast cancer metastasizes to

specific metastatic niches will help to develop effective therapeutic interventions. One potential mechanism, termed the “seed and soil” theory of metastasis suggests that the microenvironment of the pre-metastatic niche dictates where and to what extent metastasis occurs. During bone metastasis one important regulator in the bone microenvironment is osteoclasts that increase bone destruction to make room for tumor cell to invade and colonize in the bone. (Le Pape et al., 2016; Mercer et al., 2004)

Importantly, previous work from our lab has shown that tungsten accumulates in the bone and the bone is a site of long-term storage and toxicity (Bolt et al., 2016). Tungsten can alter the bone microenvironment by disrupting bone formation, regulated by osteoblasts, by skewing bone marrow MSC differentiation to increase adipogenesis and decreased osteogenesis (Bolt et al., 2016). In addition, it has been shown that tungsten exposure can increase the number of bone resorbing osteoclasts in mice treated with tungsten for 4 weeks. (Chou et al., 2021) Together, these findings demonstrate that tungsten can modulate bone homeostasis by enhancing osteoclast differentiation and or function. However, what effect these alterations in the bone microenvironment have on the potential to drive breast cancer to metastasize to the bone are unknown. Thus, in this study, we investigated the role of tungsten on breast cancer metastasis to the bone using a preclinical mouse model. We hypothesize that tungsten will enhance breast cancer metastasis to the bone by altering bone remodeling through increased osteoclast activity within the bone

microenvironment. This work provides the first experimental evidence that tungsten exposure can significantly enhance the bone metastatic potential of breast cancer cells, in mice, by stimulating a pro-tumorigenic osteolytic inflammatory microenvironment in the metastatic niche.

Methods

Chemicals and Reagents: Tungsten in the form of sodium tungstate dihydrate ($Na_2WO_4 \cdot 2H_2O$) was purchased from Sigma-Aldrich (St. Louis, Missouri). Sodium tungstate dihydrate was dissolved in tap water and replaced every 2 or 3 days to limit conversion to polytungstates.

Orthotopic Breast Cancer Model Tumor Cell Line*:* 4T1 is a triple-negative breast cancer (TNBC) cell line that lacks expression of the estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2). (Won & Spruck, 2020) The 4T1 cell line was originally isolated by Fred Miller and colleagues as one of four sublines derived from the 410.4 tumor, which is part of the Miller panel of mammary tumor cell lines, all isolated from a single spontaneously arising tumor from a BALB/c mouse (Dexter et al., 1978). Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 5% CO_2 and 37°C.

Mice: All animal experiments were performed in accordance with protocols approved by the University of New Mexico (UNM) Office of Animal Care Compliance Committee (Albuquerque, NM). Wild-type female BALB/c mice (6-8 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA) and housed in the UNM Health Science Center (HSC) Animal Resource Facility. Mice were given food and water *ad libitum*. After 1 week of acclimatization, mice were divided into 2 groups: control tap water or 15 parts per million (ppm; μg/ml) sodium tungstate dihydrate. Mice were pre-treated with and without 15 ppm sodium tungstate in drinking water for 4 weeks prior to tumor cell injection and remained exposed for the duration of the study. No changes in animal weight, physical appearance, or water intake were observed in the tungsten-exposed group. Following the pre-treatment, 4T1 tumor cells (10,000) were injected into the 4^{th} mammary fat pad of mice. Primary mammary tumor size was monitored through measurement using calipers every 3–4 days. Tumor volume was calculated using the following equation,
volume = $(4/3 \times (3.14159) \times (\text{length}/2) \times (\text{width}/2)^2)$ until the maximum tumor volume was 1500 mm^3. Once tumor-size endpoint was reached ~1500 mm^3, at ~30 days, mice were euthanized with terminal cardiac puncture. A set of non-tumor-bearing BALB/c mice was also used as treatment controls for the experiment. Non-tumor-bearing mice were given tap water or water containing 15 ppm sodium tungstate dihydrate for 8 weeks.

Blood Collection: Blood was collected by cardiac puncture from anesthetized mice. Blood was collected in EDTA-coated microcontainer tubes (Thermo Fisher Scientific, Waltham, Massachusetts) and centrifuged at 4000 g for 10 min at 4°C. Aliquots of plasma were frozen and stored at −80 C. Samples were thawed only once or twice before assay. Plasma analysis was performed by taking frozen plasma and analyzing for bone remodeling markers through ELISA assays.

Tissue Collection: Primary tumor, spleen, tibia and femur bones were excised and primary tumor and spleen were weighed. Primary tumor and spleen were weighed, fixed in 10% buffered formalin (Thermo Fisher Scientific), and paraffin-embedded (University of New Mexico Pathology Department, Albuquerque, New Mexico).

Quantification of Metastasis in the Bone: The extent of metastasis in the bone was evaluated by collecting and isolating femur and tibia bones. Next, bone marrow was extracted by removing femur and tibia heads and nesting bones within a perforated centrifuge tube, which was then nested in a collection tube. Finally, bones were centrifuged at ≥10,000 x g for 15 sec. Cells were then resuspended in media containing 60 μM 6-thioguanine and then were plated in a 6 well plate at three cell densities of 3 million, 300,000, and 30,000 cells per well. Plates were then incubated for 12 days. Cells were fixed and stained with 0.005% crystal violet. Colonies were quantified using Image J (NIH, Bethesda, Maryland) and total number of colonies were compared between groups.

Flow Cytometry: Innate immune or mesenchymal stromal cell populations were defined based on cell surface marker expression and analyzed by flow cytometry using an Attune NxT Flow Cytometer (Thermo Fisher Scientific) in both the bone marrow and blood at the end point of the study. For bone marrow, 2 batches of bone marrow cells were blocked in Fc block CD16/CD32 (BD Biosciences, Franklin Lakes, NJ) and stained with fluorescently-conjugated primary antibodies: CD11b-AF647 (BD Biosciences (BioLegend, San Diego, CA), and Ly6C-BV421 (BioLegend) to quantify the amount of granulocytic myeloid-derived suppressor cells (G-MDSCs) or CD44 (BD Biosciences) and CD29 (BioLegend) to quantify the number of mesenchymal stromal cells. For the mesenchymal stromal cell set, following staining with primary antibodies, cells were fixed and permeabilized using the fix per BD Pharmingen Transcription Factor Buffer Set (BD Biosciences), blocked with 2% rat serum and stained for the internal fibroblast activation marker α-smooth muscle actin (α-SMA) using the primary antibody (α-SMA, Abcam, Cambridge, UK) and a fluorescently-conjugated secondary antibody (anti-FITC, BioLegend). G-MDSC's were defined as CD11b+, Ly6C Low and Ly6G+. Activated mesenchymal stromal cells (MSCs) were defined as CD44+, CD29+, α-SMA+. For whole blood, cells were pelleted and washed with 1x PBS. Cells were blocked in Fc block CD16/CD32 (BD Biosciences) and stained with fluorescently-conjugated primary antibodies: CD45-BV605 (BD Biosciences), CD11b-AF700 (BD Biosciences), Gr1 (Ly6G/Ly6C)-e450 (Invitrogen eBioscience). MDSCs were defined as CD45+, CD11b+, Gr1+. Data

were analyzed using Attune NxT Analysis Software (Thermo Fisher Scientific). The percentage and/or total number of each cell type were quantified in control and tungsten-exposed 4T1 tumor-bearing mice.

Tungsten quantification in Bones: Once primary endpoint was reached tibia bones were harvest, cleaned, flushed and stored at -80 C until analysis. Tibia bones from both control and tungsten exposed 4T1 tumor-bearing mice and non-tumor controls were acid digested and tungsten concentration was quantified using PerkinElmer NexION 300D Inductively Coupled Plasma Mass Spectrometry (ICP-MS) coupled with Elemental Scientific SeaFast (Omaha, Nebraska) auto sampler using direct mode with anhydrous ammonia to significantly minimize mass interferences. Acid digestion was performed in a heat block digester using 70% trace metal grade nitric acid. Samples were heated gradually (ramp and hold) to 95C for 2 h. After digestion, the samples were normalized to a 10 ml total volume with 18M-ohm water and filtered through a 0.45 ml filter (SCP Science) prior to ICP-MS analysis. The limit of detection for tungsten was 0.050 parts per billion (ppb). The ICP-MS instrument was optimized using multi-element optimization solution over a wide range of masses and calibrated using single element tungsten standards. All solutions were made with 2% nitric acid in order to match the standards matrix. The system was calibrated with blank and 4 standards, and samples were analyzed including quality control samples for data validation and verification. The ICP-MS instrument is housed in the Analytical Chemistry Laboratory in the Department of Earth and Planetary

Sciences at the University of New Mexico. Tungsten Concentration in Bone (ppm) based on µg tungsten (W)/g tissue weight.

Bone Density: Femur bones were harvested and cleaned. One femur bone was fixed in 70% ethanol and stored at −80°C until analysis. MicroCT analysis was performed at the McGill Centre for Bone and Periodontal Research to evaluate changes in bone microarchitecture. Trabecular bone microarchitecture from control and tungsten-exposed 4T1 tumor bearing mice was analyzed using the Skyscan 1172, 50mm FOV (image field width) to produce 3D microCT images of trabecular bone. Samples were observed sticking them onto the SkyScan samples holder by using plastilin. No filter was used to scan images. Bone Surface Density measured as Bone Surface (BS)/ Total Volume (TV) (mm^2/mm^3). Percent Bone Volume measured as Bone Volume (BV)/TV was also measured along with Trabecular Number (Tb.N) measured as mm-1. Finally, Trabecular thickness (Tb.Th) measured in mm was also analyzed.

Changes to the Bone Microenvironment: To assess the effects of tungsten exposure on bone remodeling in the bone niche during breast cancer progression, femur bones were collected and either formalin fixed, and embedded in paraffin or frozen in 70% EtOH from the completed animal study described above.

TRAP Staining: Femur sections were harvested from *in vivo* tungsten-exposed mice, tissue was formalin fixed (10% buffered formalin) and decalcified in 10% EDTA. Following decalcification, tissue was embedded in paraffin and 5-μm sections were prepared on glass slides through the UNM HSC Human Tissue Repository & Tissue Analysis core. Sections were then deparaffinized in Histo-Clear (Thermo Fisher Scientific) followed by sequential alcohol rehydration to distilled water. Sections were subjected to tartrate-resistant acid phosphatase (TRAP) staining to quantify osteoclasts. Briefly, sections were incubated in incubation buffer solution containing napthol-ether substrate for 1 h at 37°C followed by incubation in a sodium nitrite-pararosaniline solution for 12 min. Sections were washed in distilled water followed by counterstaining in 0.05% Fast Green for 90 s. Sections were rinsed in distilled water followed by dehydration in sequential alcohol concentrations and Histo-Clear. Sections were mounted in Permount mounting medium (Thermo Fisher Scientific). Stained tissue sections were digitally scanned at 20x objective using the Leica Versa 200 digital scanner through the UNM HSC Human Tissue Repository & Tissue Analysis core. TRAP-positive osteoclasts were quantified using mean pixel intensity of TRAP staining utilizing Image J software Image J (NIH, Bethesda, Maryland).

ALP Staining: Femur bones were harvested from control and tungsten-exposed mice. One femur bone was fixed in 70% EtOH and stored at −80°C until embedding. Fixed, undecalcified bones were embedded in methylmethacrylate

and sectioned through the McGill Center for Bone and Periodontal Research. To assess alkaline phosphatase (ALP) enzymatic activity to quantify osteoblasts, sections of bone were stained using the Vector Red Alkaline Phosphatase Substrate Kit (Vector Labs, Burlingame, California) according to manufacturer's protocol. Sections were counter-stained with methyl green. Staining was performed through the McGill Center for Bone and Periodontal Research. Stained tissue sections were digitally scanned at 20x objective using the Leica Versa 200 digital scanner through the UNM HSC Human Tissue Repository & Tissue Analysis core. ALP-positive staining was quantified using mean pixel intensity of ALP staining utilizing Image J software Image J (NIH, Bethesda, Maryland).

RNA Isolation and RT-qPCR: The assessment of messenger RNA (mRNA) expression was quantified by RT-qPCR using previously published methods. (Aragon et al., 2017; Garcia et al., 2021) In brief, total RNA, from bone marrow cells, was extracted using the RNeasy Mini Kit (Qiagen, Germantown, Maryland). RNA (500 ng by concentration) was reverse-transcribed with random primers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California) following the manufacturer's protocol. To quantify gene expression, cDNA was amplified for multiple gene targets by real-time qPCR (RT-qPCR) using a LightCycler 480 Instrument II (Roche, 05015278001). All primers and probe sets were purchased as predesigned validated TaqMan Gene Expression Assays (Applied Biosystems). Genes of interest (GOI) included: Acta-

2 (Mm01546133_m1), SDF1 (Mm00436450_m1), TGFB-1 (Mm01178820_m1), OPN (Mm00436767_m1), OPG (Mm00437135_m1), RANK-L (Mm00441906_m1), MMP1 (Mm00473485_m1), IL-11 (Mm00434162_m1), TNF-α (Mm00443258_m1), Hif-1a (Mm00468869_m1), IL-1β (Mm00434228_m1), and MMP9 (Mm00442991_m1). Relative expression of GOI was determined by the comparative threshold method ($2^{-\Delta\Delta CT}$) and normalized to Rn18s housekeeping gene (Mm03928990_g1). Data were expressed as fold change in expression relative to the tap water group. All samples were analyzed in triplicate.

Analysis of Bone Remodeling Markers in Plasma: Frozen plasma, collected from control and tumor-bearing mice, was analyzed for markers of bone remodeling PINP and TRACP 5b by Enzyme-linked Immunosorbent Assay (ELISA). The concentration of bone remodeling markers in plasma was determined using commercially available ELISA kits (PINP; Immunodiagnostic systems and TRACP 5b; Immunodiagnostic systems) according to manufacturer's protocols. The detection limits for PINP and TRACP 5b were 0.7 – 75 ng/ml and 0.3-10.0 U/L, respectively. All samples were analyzed in duplicate.

Changes to 4T1 Cells in the Presence of Tungsten: To assess the direct effects of tungsten on 4T1 breast cancer cells, we evaluated expression of genes that drive tumor cell colonization and growth at the metastatic niche using RT-qPCR. 4T1 cells were incubated in four separate groups: control, 3, 5, and 10 µg/ml tungsten. Briefly, 50,000 cells were plated into a 6 well plate, and allowed to

attach for roughly 6 hours before treating with the sodium tungstate. Stock solution was mixed using serial dilutions to 1ug/ul in MilliQ water, which was added directly into the wells. Cells were then allowed to grow for 3 days, on day 3 cells were collected, pelleted and froze before RNA extraction. After which cells were harvested and RNA was extracted was isolated using the RNeasy Mini Kit (Qiagen, Germantown, Maryland). RNA (500 ng by concentration) was reverse-transcribed with random primers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California) following the manufacturer's protocol. qPCR was then run on all groups utilizing same protocol mentioned in above section to determine fold expression of the known factors secreted or upregulated by breast cancer cells in the bone microenvironment. These include: Acta-2, IL-1β, MMP9, OPN.

Statistics: All statistics were performed using GraphPad Prism version 9 software (GraphPad, La Jolla, California). For comparisons between 2 groups, an unpaired Student's t-test analysis was performed. For comparisons between 3 or more groups, either a 1-way or 2-way ANOVA analysis was performed based on the number of continuous variables analyzed. Outliers to data sets were removed by performing a Grubb's Outlier Test ($p\text{-value} \leq 0.05$). All data were graphed as mean ± standard error of the mean (SEM), and individual data points for each experimental group. The significance level used in this study to determine statistically relevant changes between groups was set to $p\text{-value} \leq 0.05$.

Results

Tungsten Does Not Alter 4T1 Primary Tumor Growth.

At the end point of the experiment, tumors were excised and weighed. Measured final tumor weight was recorded in grams and analyzed. Our data (Figure 2A) shows that final tumor weight (g) was not affected by oral tungsten exposure of 15ppm. To evaluate the effect of tungsten exposure on primary breast tumor growth throughout tumor progression, tumor measurements were made weekly as described in the methods section previously. Tumor measurements taken throughout the duration of the experiment were also similar, suggesting that tungsten exposure did not affect primary breast tumor growth (Figure 2B). These findings build on previously shown data, suggesting that tungsten has no effect on primary mammary tumor growth using multiple orthotopic triple negative breast cancer mouse models.

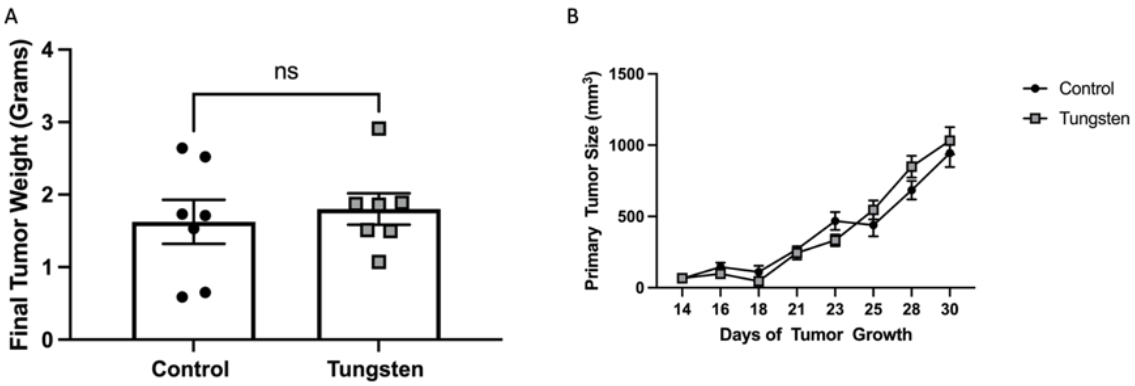

Figure 2. Tungsten does not alter 4T1 primary tumor growth throughout tumor progression. *A, shows final tumor weight in grams. Oral tungsten (15 ppm) exposure did not affect primary tumor growth. B, graphs show Primary Tumor Size (mm3) verses Days of Tumor Growth and Final Tumor Weight (g) for control and tungsten-exposed 4T1 tumor bearing mice.*

Tungsten Accumulation in the Bone is Slightly Reduced in 4T1 Tumor-Bearing Mice Compared to Non-Tumor-Bearing Controls.

The bone is a target of tungsten toxicity due to its ability to accumulate and chronically release tungsten over time. (Bolt et al., 2016; Bolt et al., 2015; Kelly et al., 2013) Tungsten burden in the bone was measure by ICP-MS in control and tungsten-exposed 4T1 tumor-bearing mice. Tungsten accumulation was increased in mice exposed to tungsten through their drinking water (Figure 3). Almost a third of the oral drinking water dose of tungsten (15 ppm) accumulates in the bone following ~8-weeks of exposure. In addition, 4T1 tumor-bearing mice were compared to non-tumor bearing mice exposed to 15 ppm tungsten, orally for 8 weeks. Interestingly, there was a slight, albeit significant decrease in the concentration of tungsten found in the bones of the tungsten-exposed 4T1 tumor-bearing mice compared to the tungsten-exposed non-tumor controls. However, the exact mechanism driving this slight decrease in tungsten concentration within tumor-bearing mice has not been explored and may not be biologically significant. These findings suggest that the bone niche is a site of tungsten accumulation and a relevant site to investigate the effects of tungsten on breast cancer metastasis.

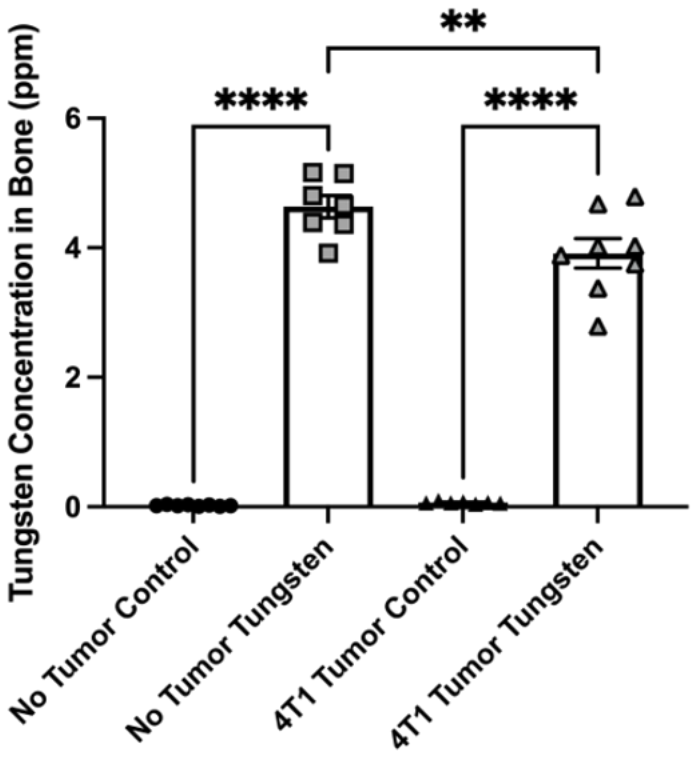

Figure 3. Tungsten accumulation in the bone is reduced in 4T1 tumor-bearing mice compared to non-tumor-bearing controls. *Tungsten concentration was recorded as 0.0659 ppm in the No Tumor Control (CTL) group receiving tap water and 4.635ppm in the No Tumor Tungsten (W) group receiving 15 ppm sodium tungstate in drinking water. Similar levels of tungsten were observed in the 4T1 Tumor Control group (0.0205 ppm). Interestingly, tungsten concentrations were slightly lower in tungsten-exposed 4T1 tumor-bearing mice (4T1 Tumor Tungsten; 3.9148 ppm) compared to WT BALB/c mice exposed to 15 ppm sodium tungstate for the same duration of time. **p-value ≤ 0.01; **** p-value ≤ 0.0001 one-way ANOVA.*

Tungsten Exposure Increased 4T1 Metastasis to the Bone.

We have previously shown that tungsten can enhanced breast cancer progression by enhancing metastasis to the lungs (Bolt et al., 2015). Breast cancer metastasis can progress to other sites including the bone. Tungsten accumulation and toxicity in the bone has been investigated in previous studies (Bolt et al., 2015; Miller et al., 2021), but there is no data evaluating how exposure to tungsten influences breast cancer metastasis to the bone and the bone microenvironment. We hypothesized that tungsten will enhance breast cancer metastasis to the bone by altering bone remodeling through increased osteoclast activity within the bone microenvironment.

Analysis of H&E tissue sections of femur bones of control and tungsten-exposed 4T1 tumor-bearing mice did not reveal large bone metastases by visual inspection, suggesting only micro metastases were present (Data not shown). In order to quantify the extent of bone micro metastases in control and tungsten-exposed 4T1 tumor-bearing mice we used bone marrow colony assays using the selection agent 6-thioguanine. Interestingly, the percentage of mice with detectable bone metastases was significantly increased in the tungsten-exposed group. The total number of mice with at least one 4T1 positive clone was greater in the tungsten group, 100% versus 37.5% in the control group mice. (Figure 4C). In addition, there was a significant increase in the total number of 4T1 positive colonies per mouse within the tungsten group, even after averaging for the total

volume of the final tumor at sacrifice (mean of 3.097 colonies for the control group and a mean of 54.08 colonies for the tungsten group; Figure 4D). These findings suggest that tungsten plays a significant role in increasing metastasis to the bone and lead us to explore possible mechanisms involved in this pathway.

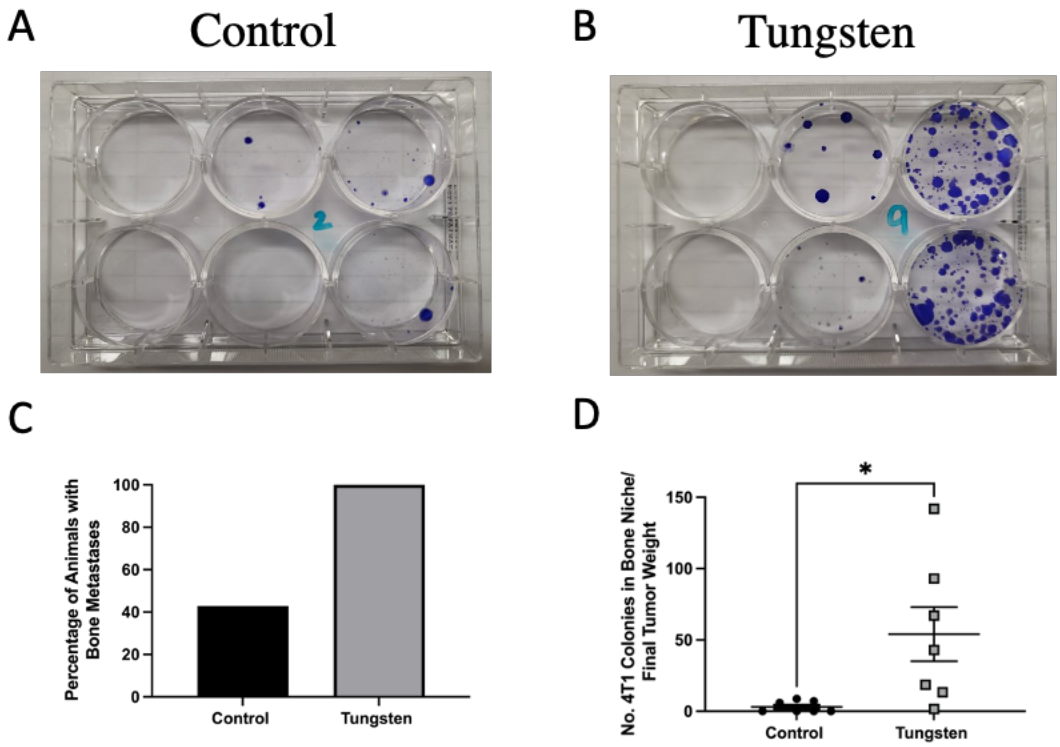

Figure 4. Tungsten exposure increased 4T1 metastasis to the bone. *A, B, the number of 4T1 colonies present after 12 days of culture were quantified. 4T1 colony number was significantly increased in the tungsten group. C, the total number of mice with any metastasis in the bone was greater in the tungsten group (n=7) versus the control group (n=3) mice. Therefore, tungsten plays some role in increasing metastasis to the bone. D, finally, when number of colonies was averaged for the total volume of the final tumor at sacrifice a mean of 3.097 colonies for the control group and a mean of 54.08 colonies for the tungsten group. This number was also significantly elevated for the tungsten group. * p-value ≤ 0.05 student t-test. This elevated number of colonies reliably suggests that there was an increased number of 4T1 breast cancer cells present within the bone marrow which is a hallmark of metastasis to the bone.*

Tungsten Enhances Breast Cancer Trabecular Osteolysis.

Often, in order for tumor cells to growth within the bone niche, osteolysis needs to occur to create space for tumor expansion. To determine if tungsten exposure and metastasis in the bone results in osteolysis, we evaluated multiple markers of bone microarchitecture from our *in vivo* experiment. Femur bones were removed and sent for microCT analysis to evaluate trabecular bone microarchitecture from control and tungsten-exposed 4T1 tumor bearing mice. 3D microCT images of trabecular bone were examined (Figure 5A). Interestingly, micro-CT analysis performed on femur bones harvested from 4T1 tumor-bearing mice, showed a marked increase in bone osteolysis in the tungsten treated mice compared to control mice, including decreases in bone surface density (Figure 5B), bone volume/total volume ratio (Figure 5C), trabecular bone number (Figure 5D), and an increase in trabecular bone separation (Figure 5F). Of note no change to trabecular thickness was noted (Figure 5E). Together these results suggest bone remodeling processes are disrupted leading to enhanced bone degradation. This degradation often defined as bone osteolysis is an important hallmark of metastasis to the bone niche.

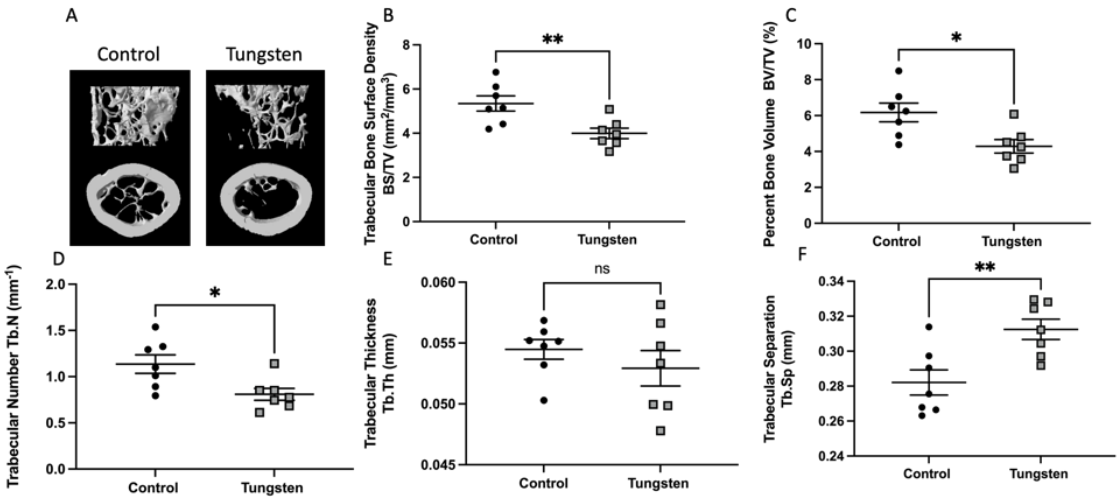

Figure 5. Tungsten enhances breast cancer trabecular osteolysis. *A, images of 3D MicroCT bone scans from femur bones of control and tungsten-exposed 4T1 tumor bearing mice. B, Bone Surface Density measured as Bone Surface (BS)/ Total Volume (TV) (mm^2/mm^3) was significantly reduced in tungsten versus control, 3.996 versus 5.345 mm^2/mm^3 respectively. C, Percent Bone Volume measured as Bone Volume (BV)/TV was significantly reduced in tungsten versus control, 4.3 versus 6.2 % respectively. D, Trabecular Number (Tb.N) measured as mm-1 was significantly reduced in tungsten versus control, 0.809 versus 1.139 mm-1 respectively. E, Trabecular thickness (Tb.Th) measured in mm appeared similar between both groups with 0.053 versus 0.055 mm. F, Finally, Trabecular Separation (Tb.Sp) also measured in mm was significantly increased in tungsten versus control 0.313 versus 0.282 mm respectively. * p-value ≤ 0.05; ** p-value ≤ 0.01 student t-test per parameter.*

Tungsten-Mediated Osteolysis Was Not Reflected in Observable Changes in Osteoclasts or Osteoblasts.

The changes to bone microarchitecture in our *in vivo* experiment were of great concern in conjunction with enhanced metastasis number. It has been previously demonstrated that tungsten treated animals have an increase in the number of osteoclasts per area of trabecular bone follow 4 weeks of oral exposure. (Chou et al., 2021) Therefore, we wanted to investigate the possible role of changes to bone microenvironment by evaluating the numbers of osteoclasts and osteoblasts in the bone niche. TRAP-positive osteoclasts and ALP-positive osteoblasts were quantified using mean pixel intensity utilizing Image J software. Overall, we saw no discernable changes in osteoblast or osteoclast staining between control and tungsten groups (Figure6A,B), which would suggest that tungsten is not enhancing osteoblast/osteoclast number or activity in order to drive breast cancer osteolysis at the time point. Results were further reflected in plasma levels of specific bone remodeling markers as no discernable differences were identified (Figure 7A,B). TRAP 5b enzymatic levels, a marker of osteoclast activity, were quantified via ELISA with no statistical changes between control and tungsten groups. Similarly, there was no appreciable change between control and tungsten groups in P1NP levels, a highly specific bone turnover marker (Jensen et al., 2002), also quantified via ELISA.

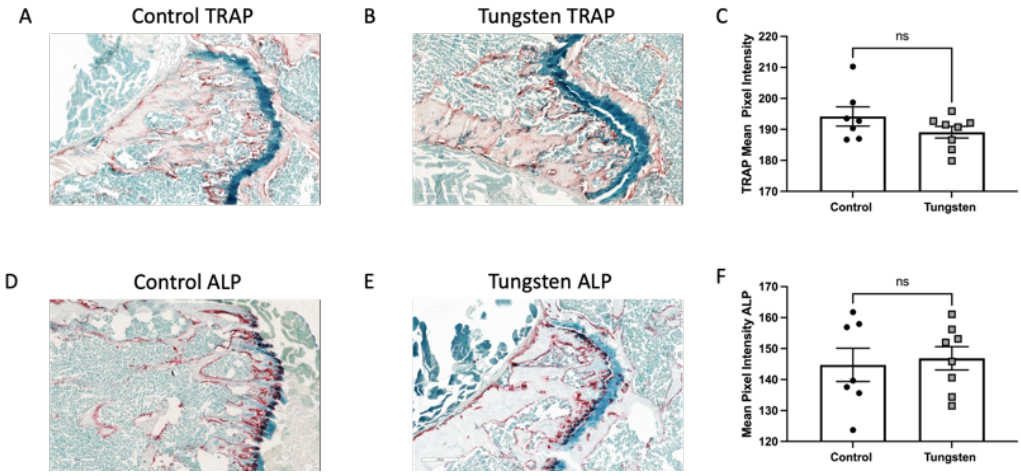

Figure 6. Tungsten does not change observable osteoblast or osteoclast number. *A,B show images of TRAP staining under the femur growth plate of control and tungsten-exposed 4T1 tumor-bearing mice, 10x objective C, there was no appreciable change between control and tungsten groups. Graph represents the number of TRAP-positive osteoclasts, which were quantified using staining mean pixel intensity utilizing Image J software. D,E show images of ALP staining under the femur growth plate of control and tungsten-exposed 4T1 tumor-bearing mice, 10x objective. F, similarly, graph represents the number of ALP-positive osteoblasts, which were quantified using staining mean pixel intensity utilizing Image J software. There was no appreciable change between control and tungsten groups.*

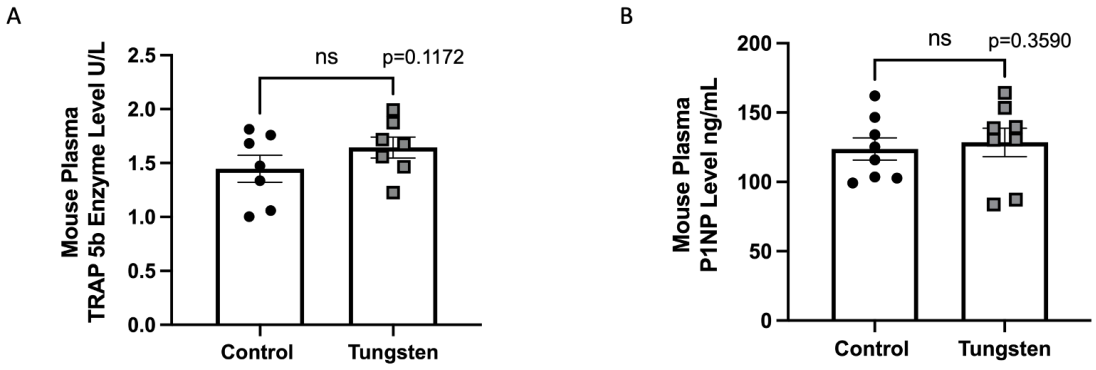

Figure 7. Tungsten does not alter plasma bone remodeling markers. *A, there was no appreciable change between control and tungsten groups in mouse plasma marker for TRAP 5b, a marker of osteoclast activity. Graph shows enzyme levels of TRAP 5b in the plasma quantified via ELISA. B, similarly, there was no appreciable change between control and tungsten groups in P1NP levels. Graph shows P1NP levels in the plasma, a specific bone turnover marker, which were quantified via ELISA.*

Tungsten Drives Breast Cancer Metastasis by Creating a Pro-Tumorigenic Immune Suppressive Environment in the Bone.

Next, we postulated if tungsten could alter the bone microenvironment making it more hospitable for tumor cells to colonization and grow in the bone niche by increasing tumor homing and proliferation factors. MDSCs are immune suppressive and play a role in tumor maintenance and progression. MDSCs also obstruct therapies that seek to treat cancer through both immunotherapy and other non-immune means. (Ostrand-Rosenberg, 2021) Activated myofibroblasts are the predominant source of production of collagens needed to form the scar and express contractile proteins which includes αSMA. In carcinogenesis, which can act as a non-healing wound, myofibroblasts can be deficient or fulfill other functions such as the production of proinvasive proteinases. (Basset et al., 1990) We have previously shown an increase in the number of G-MDSCs in the periphery and the lungs and the number of activated myofibroblasts in the lungs of 66Cl4 breast cancer tumor-bearing mice following tungsten exposure. (Bolt et al., 2015)

To this end monocytic and G-MDSCs and activated myofibroblasts were analyzed in the bone marrow by flow cytometry using fluorescently tagged antibodies. Tungsten increased the number of both G-MDSCs and activated myofibroblasts (Figure 8A,B), but did not increase the number of

monocytic MDSCs (data not shown). Of note myofibroblasts are the predominant source of production of collagens needed to form the scar and express contractile proteins which includes αSMA. Furthermore, the process of conversion from cardiac fibroblasts to myofibroblasts has been shown to be medicated by TGF-B. Therefore, control of the inflammatory and repair pathways could be required to prevent an overactive inflammatory response. (Sun et al., 2021)

Furthermore, upregulation of fibrotic markers such as Acta-2 are often associated with the activity of the fibrotic process, and are thought to indicate the extent of connective tissue deposition. This is pertinent as G-MDSCs induce inflammatory phenotypes and have been shown to upregulate fibrotic markers (Col3a1, Postn, Acta-2), fibrosis-related factors (MMP9), and inflammatory cytokines (IL-6 and IL-10) levels in fibroblasts. (Sun et al., 2021) We profiled a panel of pro-inflammatory and premetastatic markers by RT-qPCR from bone marrow isolated from control and tungsten-exposed 4T1 tumor-bearing mice. As expected we observed an increase in Acta-2, IL-1β, TNF-a, and Hif-1a (Figure 9A-D) through quantification utilizing RT-qPCR. Of note OPN ($p=0.069$), SDF1 ($p=0.063$), and MMP9 ($p=0.0743$) were approaching significance (Figure 9E-F). In addition, markers were also quantified that failed to reach or approach significance. These included: TGFβ-1, Runx2, OPG, and RANK-L (Data not shown). This further characterization of the bone

microenvironment along with previous data supporting osteolysis suggests that the etiology behind increased bone metastasis following tungsten exposure is multifactorial.

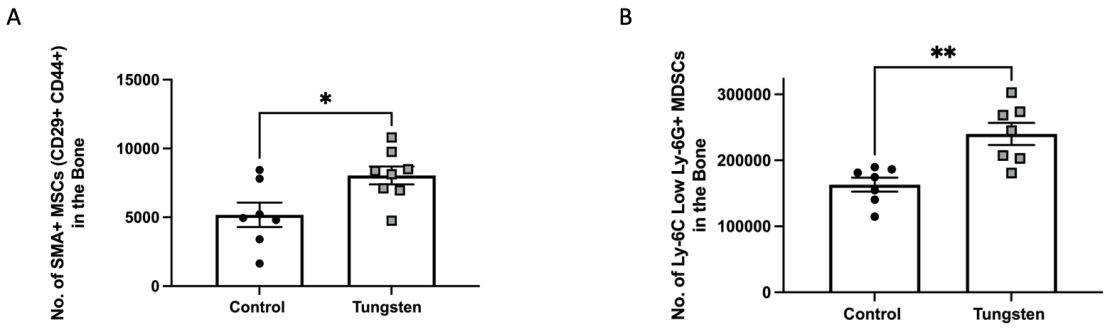

Figure 8. Tungsten increases the number of both granulocytic MDSCs (G-MDSCs) and activated myofibroblasts in the bone niche.

*A, Number of activated myofibroblasts were identified as those cells with the following markers: SMA positive, CD29 positive, and CD44 positive. B, Number of G-MDSCs were identified as those cells with the following markers: Ly-6C low, Ly-6G positive, and CD45 positive. The expression of pro-tumorigenic gene in the bone marrow were then profiled by flow cytometry. * p-value ≤ 0.05, ** p-value ≤ 0.01, student t-test per parameter.*

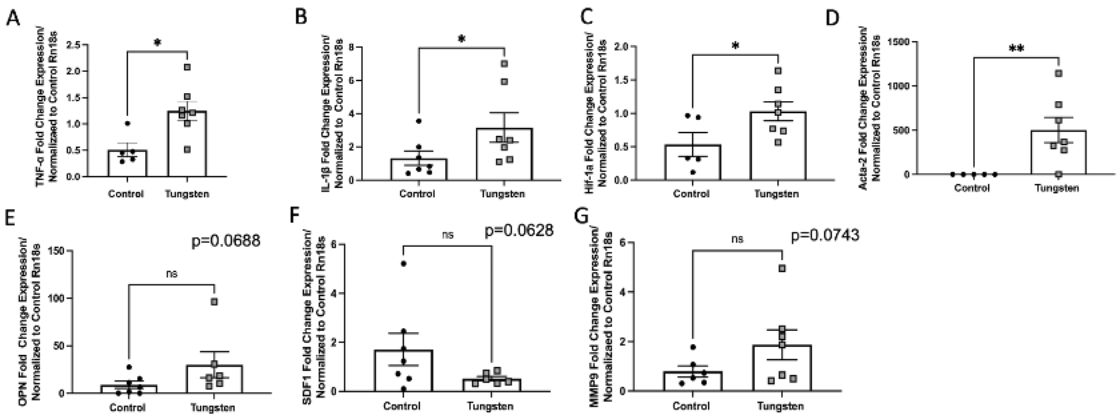

Figure 9. Tungsten alters the microenvironment in the bone niche to promote a pro-metastatic environment. *A,TNF-a is promoter of inflammatory responses, was increased in the bone marrow in tungsten exposed mice. B, IL-1β, is another pro-inflammatory cytokine that also signals homing to the bone and metastatic growth. C, Hif-1a overexpression is implicated in promoting tumor growth and metastasis through initiation of angiogenesis and regulating cellular metabolism to overcome hypoxia. D, Furthermore, the activated fibroblast marker α-smooth muscle actin (Acta-2) known for enhances distant metastasis, promotion of tumor cell proliferation, and inhibited apoptosis in breast cancer was found significant. * p-value ≤ 0.05, ** p-value ≤ 0.01 student t-test per parameter (one-tailed). E-F, Of note OPN, SDF1, and MMP9 were approaching significance.*

Tungsten Exposure Did Not Affect Spleen Size or Tumor Immune Response in the Peripheral Blood.

Given the changes to inflammatory cells and cytokines in the bone niche following tungsten exposure, we wanted to see if these changes in inflammatory endpoints were reflected in the peripheral blood and spleen. The number of CD45+, CD11b+, Gr1+ MDSCs in peripheral blood were quantified using multi-parameter flow cytometry with fluorescently tagged antibodies. Overall, no changes were observed in the Number of Cells/mL Blood or the Percent CD45+ CD11b+ Gr1+ MDSCs in control and tungsten-exposed 4T1 tumor-bearing mice. (Figure 10A,B). Spleen weight was measured directly after mice were sacrificed in control and tungsten-exposed tumor mice. Again, there was no appreciable difference between control and tungsten groups (Figure 10C). These results suggest that while an observable difference within immune endpoints occurs within the bone niche, there is little evidence of systemic immune, emphasizing the selective impact on the bone.

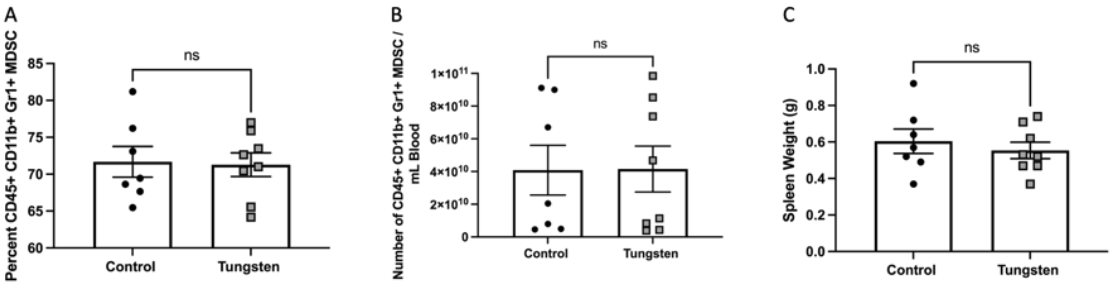

Figure 10. Tungsten exposure did not affect spleen size or tumor immune response in the peripheral blood. *The percentage and number of CD45+, CD11b+, Gr1+ myeloid derived suppressor cells (MDSCs) in peripheral blood were quantified using multi-parameter flow cytometry using fluorescently tagged antibodies. A, the Percent of Cells/mL Blood and B, the Number of CD45+ CD11b+ Gr1+ MDSC in control and tungsten-exposed 4T1 tumor-bearing mice. C, Total Spleen Weight in grams in control and tungsten-exposed mice.*

Tungsten Increases Expression of Inflammatory and Pro-metastatic Markers in 4T1 Cells *In vitro*.

Given the changes observed *in vivo* within the bone niche we sought to start to evaluate if these changes in pro-inflammatory, premetastatic markers were coming from cells in the bone marrow or from the tumor cells. We evaluated 4 of the markers by RT-qPCR (Acta-2, IL-1β, MMP9, and OPN) in 4T1 cells with and without treatment with increasing concentrations of tungsten for 3 days *in vitro*. Interestingly, increasing concentrations of tungsten resulted in an increase in the expression of all 4 genes *in vitro* (Figure 11). This further characterization of the 4T1 cells along with previous data supporting changes within the bone microenvironment suggests that the etiology behind increased bone metastasis following tungsten exposure could be complicated and more work will be required to determine the direct verses indirect effects of tungsten to drive breast cancer metastasis to the bone niche.

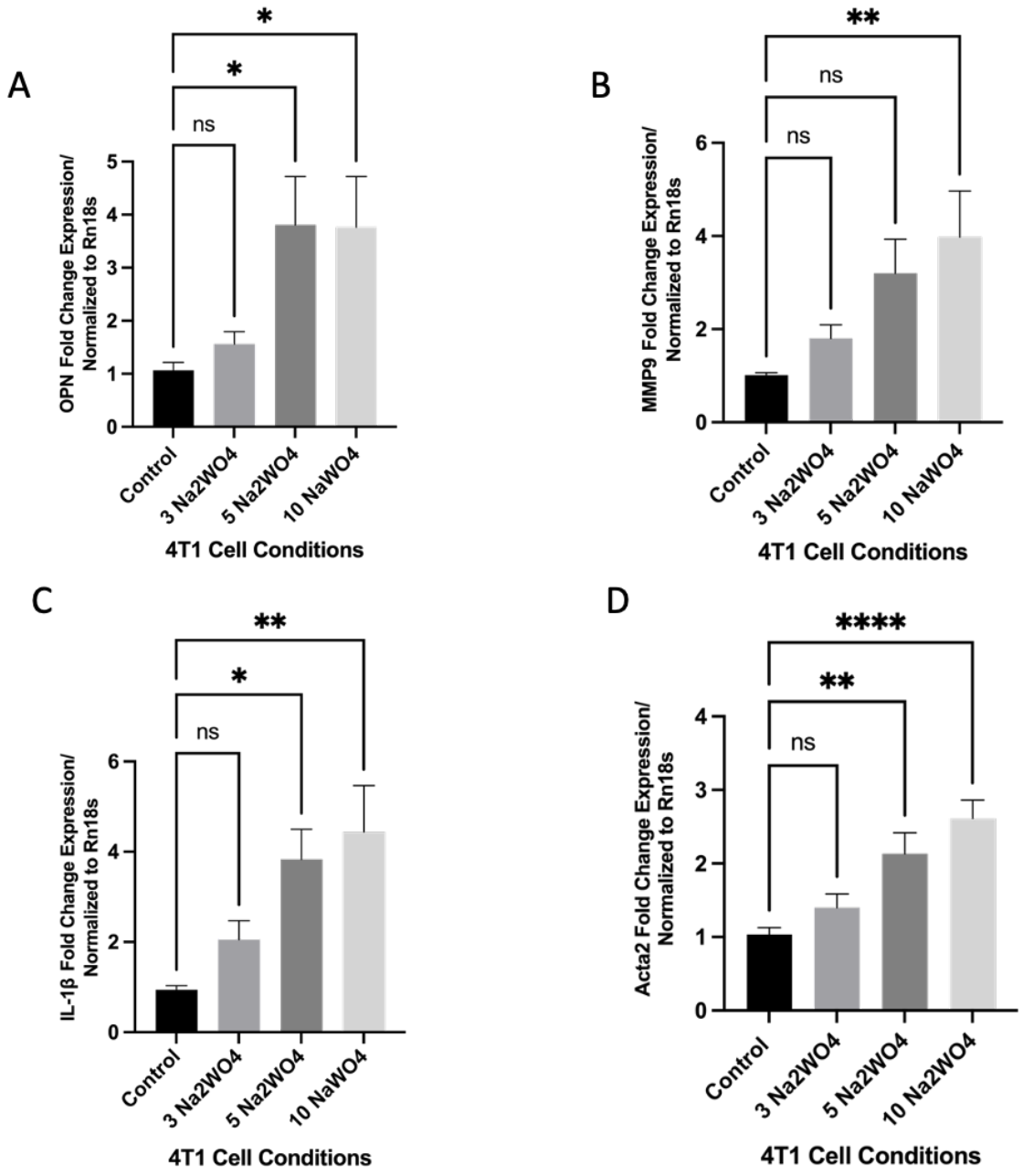

Figure 11. In vitro tungsten exposure increases expression of inflammatory and pro-metastatic markers in 4T1 cells. *Changes in the expression of target genes were measured by RT-qPCR using validated Taq-man primers. 4T1 cells were treated with increasing concentrations of NaWO4 (µg/mL) in vitro for 3 days. Graphs show the Fold Change*

*Expression of genes A.OPN, B. MMP9, C. IL-1β, and D. Acta2, normalized to the housekeeping gene Rn18s. * p-value ≤ 0.05 one-way ANOVA compared to Control group for each parameter. Data reflective of replicates from 3 independent experiments.*

Discussion and Conclusions

It has been previously established that tungsten exposure drives breast cancer progression by enhancing metastasis to the lungs using an aggressive orthotopic model of triple negative breast cancer (Bolt et al., 2015). However, the bone is also an important site of breast cancer metastasis and also a known site of tungsten storage and toxicity. We sought to investigate if tungsten accumulation in the bone leads to enhanced breast cancer metastasis to the bone niche. We were able to establish that tungsten increased metastasis to the bone by plating bone marrow cells flushed from the femur bones in the presence of 6-thioguanine. These results showed a greater than ten-fold increase in colonies present within the tungsten groups, and led us to question what could be causing such a marked increase in metastasis.

Therefore, we sought to test several factors to determine what changes tungsten imparts in the bone microenvironment in order to drive metastasis to the bone niche. These factors were split up into two main groups to determine how factors contributed to the metastatic process as defined in the seed and soil hypothesis. Of these factors we examine bone turnover and how it applies to the amount of physical space within the bone niche as well as changes in the bone microenvironment that lead to

enhanced tumor cell homing and colonization and growth within the bone niche.

Through the use of microCT analysis we were able to established that breast cancer osteolysis of the trabecular bone was occurring to a greater degree in the bones of tungsten-treated tumor-bearing mice as compared to those treated with tap water alone. Bone osteolysis is an important hallmark of metastasis to the bone niche where bone remodeling processes are disrupted leading to enhanced bone degradation creating space for tumor colonization and growth. (Todd & Johnson, 2020) Therefore, this increase to physical space within the bone suggests that one of the factors contributing to increased metastasis within tungsten-treated mice is increased osteolysis. Previous research done in tungsten-exposed mice observed significantly elevated osteoclast numbers in the trabecular bone of femurs following oral exposure to tungsten in adolescent male, but not female mice. (Chou et al., 2021) Tungsten also enhanced RANKL-induced differentiation into TRAP-positive mononucleated osteoclasts *in vitro*. Importantly, tungsten alone had no effect on differentiation or on the number of multinucleated TRAP-positive osteoclasts. (Chou et al., 2021) This led us to quantify the number of TRAP positive osteoclasts in our mice and elaborate on their possible role in the development of osteolysis. However, calculating mean intensity over area there was no difference between groups. These results led us to

investigate osteoblast activity in the bone as another potential target for the cause of osteolysis. In this case, ALP staining is often utilized to quantify osteoblast number/activity within the bone microenvironment. However, again calculating mean intensity over area there was no appreciable difference of note in trabecular or cortical bones. Furthermore, results were further reflected in plasma levels of specific bone remodeling markers as no discernable differences were identified. In the case of TRAP 5b enzymatic levels, a marker of osteoclast activity, were quantified via ELISA with no statistical changes observed between control and tungsten groups. Similarly, there was no appreciable change between control and tungsten groups in P1NP levels, a highly specific bone turnover marker (Jensen et al., 2002), also quantified via ELISA. Based on these results at this time point it does not appear that tungsten is changing the number or activity of osteoclasts or osteoblasts in the bone. However, a more careful analysis of bone remodeling markers throughout tumor progression might be required to determine how tungsten might be affecting these cell types in the pathogenesis of enhanced breast cancer osteolysis. We also plan to explore other fundamental mechanisms of osteolysis mediated through cytokine signaling through the use of flow cytometry and RT-qPCR to establish which factors may be contributing to this process.

We have demonstrated that tungsten not only changes the physical microenvironment, but also causes changes to the immune response in the bone niche that can drive metastasis including increased numbers of G-MDSCs, increased numbers of activated MSCs, and increased levels of pro-inflammatory/pro-metastatic markers TNF-a, IL-1β, Acta-2 and Hif-1a. We have previously shown that tungsten-enhanced breast cancer metastasis to the lungs was driven by an increase in G-MDSCs and Cancer-Associated Fibroblasts in the lung niche as well as an increase in the number of G-MDSCs and levels of MMP9 in the periphery. (Bolt et al., 2015)

Together, these factors identify an area of concern in the role of tungsten in metastasis. Of note MDSCs contribute to a pro-tumorigenic environment as they suppress T-cell and NK-cell activity to promote tumor growth (Tumino et al., 2021) and were also shown to have a marked increase in tungsten-bearing mice within the bone marrow. Interestingly Gr1+ MDSCs have been previously demonstrated to be increased in response to breast cancer metastasis to the lungs following tungsten exposure.(Bolt et al., 2015) One could hypothesize that altering the balance of MDSCs in conjunction with increased levels of pro-metastatic factors such as Acta-2 and IL-1β, known to enhance distant metastasis, promote tumor cell proliferation, and inhibited apoptosis in breast cancer

(Sun et al., 2021; Tulotta et al., 2019), may be the primary driving factors behind enhance bone metastasis following tungsten exposure.

Together, our data identifies the bone as a target of tungsten toxicity and provides evidence that tungsten deposition in the bone increases breast cancer metastasis to the bone. However, what the primary driving factors regulating enhanced metastasis and osteolysis in the bone following tungsten exposure have yet to be fully understood. However, tungsten's negative correlation especially in the presence of breast cancer further justifies its status as an emerging toxicant. Therefore, in the future we want to determine molecular drivers of how tungsten is inducing this inflammatory pro-metastatic environment in the bone niche. Furthermore, by delineating the direct effects of tungsten on dormant breast cancer cells versus the indirect effects of tungsten on the microenvironment in the bone niche to drive metastasis we may be able to better understand the underlying factors behind these mechanisms

Interestingly, although we did observe marked changes in the bone niche that resulted in an inflammatory pro-metastatic microenvironment, we did not observe changes in immune responses in either the peripheral blood or the spleen. This is contrary to what was observed previously using the 66Cl4 orthotopic breast cancer model where we did see a significant increase in the number of MDSCs in the peripheral blood and

enlarged spleen following tungsten exposure. (Bolt et al., 2015) It is difficult to determine why these effects were not replicated in the 4T1 orthotopic model, but one reason could be how aggressive this model is and how fast progression occurs. In order to fully evaluate the effects of tungsten on the peripheral immune response we might need to look at the immune responses in the periphery more sequentially throughout tumor progression instead of just at the endpoint of the study.

As mentioned previously, given the changes observed *in vivo* within the bone niche we sought to start to evaluate if these changes in pro-inflammatory, pro-metastatic markers were coming from cells in the bone marrow or from the tumor cells. Our *in vitro* data highlights that tungsten may also have a direct effect on tumor cells once they have metastasized to the bone niche that leads to enhanced colonization and growth. Therefore, in the future it will be important to fully characterize the effects of tungsten on the tumor cells versus the bone metastatic microenvironment. In order to determine the etiology behind tungsten driven enhanced breast cancer metastasis to the bone niche.

In this study we demonstrated that exposure to tungsten, in the drinking water of mice at a level of 15 ppm, resulted in tungsten accumulation within the bone and several notable changes in the microenvironment within the bone niche including increased osteolysis

and an increase to pro-tumorigenic inflammatory cytokine signals. Our study continues to suggest that the alterations caused by chronic tungsten exposure in the bone niche could be important drivers of disease pathogenesis including enhanced breast cancer tumorigenesis and metastasis. Importantly, our data also show that tungsten affects the bone in more ways than originally defined. We have reliably shown data that suggests that promotion of metastasis following tungsten exposure is multifactorial. This should be of great concern as breast cancer metastasis to the bone is severe and often identified in advanced stages of breast cancer. Therefore, our data relating oral tungsten exposure to worsening disease progression is of great concern.

Chapter 3: Discussion and Conclusions

Discussion of Results

Although tungsten exposure has yet to be recognized as a human health hazard to the majority of the United States, it remains a subject of intense concern as it is still identified as an emerging environmental toxicant. One area of concern is the limited evidence of the potential

carcinogenic/tumorigenic effects of tungsten. Multiple *in vivo* studies have identified that tungsten exposure can accelerate tumor progression and drive metastasis. (Kalinich et al., 2005; Wei et al., 1987) Our lab has been investigating the effects of tungsten exposure on breast cancer progression due to a cohort of breast cancer patients accidently exposed to tungsten through the use of a tungsten-based shield used during intraoperative radiotherapy. (Bolt et al., 2015)

Based on strong preliminary data we know that the bone is a long-term storage site for tungsten and is also a primary site of breast cancer metastasis in advanced disease. The principal aim of this study was to assess breast cancer metastasis to the bone following tungsten exposure *in vivo*. I strongly believe that my work has further advanced our understanding of the effects of tungsten-exposure on breast cancer progression and has identified that tungsten targets the bone niche in disease pathogenesis. This project focused on two separate parts. First to assess breast cancer metastasis to the bone following oral exposure to tungsten *in vivo*. Second to evaluate makers of bone remodeling in the bone of tumor-bearing mice following tungsten exposure *in vivo*.

We first needed to establish that tungsten did accumulate in the bone in our model. We did this through the use of ICP-MS where we showed that mice who received tap water only had a tungsten level of 0.0659 ppm

in their bones versus the mice treated with 15 ppm sodium tungstate for the same duration of time had a tungsten level of 3.9148 ppm ($p \leq 0.0001$). This established that our treatment using sodium tungstate in the drinking water led to an appreciable increase in tungsten concentration within the bone. This oral drinking water exposure to tungsten is higher than what was detected in the breast cancer patients exposed to tungsten, but is reflective of systemic exposure concentrations that occur in occupational and environmental settings. (Rubin et al., 2007; Schell & Pardus, 2008)

We used a well-established model of triple negative breast cancer utilizing 4T1 tumor cells injected directly into the mammary fat pad. Importantly this model is known to metastasize to the bone, which is why it was chosen for our studies. For this model we utilized 4T1 breast cancer cells and similarly to our previous studies done with the 66Cl4 orthotopic model, oral tungsten exposure did not alter primary mammary tumor growth in the 4T1 orthotopic breast cancer model as final tumor weights at sacrifice were similar between groups.

Our research demonstrated a novel discovery. We established that following tungsten exposure there was a distinct and dramatic increase in the number of metastases present within the bone microenvironment of the 4T1 tumor-bearing mice compared to control. This provides strong

evidence to support Aim 1 of this thesis where we set out to assess breast cancer metastasis to the bone following oral exposure to tungsten *in vivo*. Interestingly, while the experimental mouse model utilized for our studies was experimentally identical to previous work in the lab using the 66Cl4 orthotopic breast cancer model, not all data from that original study was replicated. In the 66Cl4 model the Bolt lab observed that tungsten enhanced breast cancer metastasis to the lungs. In the 4T1 we did not see a significant increase in lung metastasis following tungsten exposure (Data not shown). One possibility for this discrepancy is due to the aggressive nature of the 4T1 cell line. Therefore, we may have not been able to detect changes in the lungs because of how rapidly metastasis occurred. In the future it may be possible to observe mice at an earlier time point during tumor progression or to remove primary tumor again at an earlier period during progression in an attempt to detected changes in lung metastases following tungsten exposure using the 4T1 orthotopic model.

Therefore, with the data above we aimed to address our second Aim, “Evaluate makers of bone remodeling in the bone of tumor-bearing mice following tungsten exposure *in vivo*.” Again, it is of my opinion that I believe we have sufficiently addressed this aim within our research.

Appreciating that breast cancer metastases is a complex disease state that affects many aspects of the body, our first goal was to establish how it may affect the bone specifically. Referring back to one potential mechanism, termed the “seed and soil” theory of metastasis suggests that the microenvironment of the pre-metastatic niche dictates where and to what extent metastasis occurs. It is believed that this microenvironment is primed by invading tumor cells, which serve to further enrich the pre-metastatic niche (Le Pape et al., 2016; Mercer et al., 2004) Therefore, our investigations aimed at the investigation of the pre-metastatic niche through various markers of bone microarchitecture, and tumor cell homing and proliferation markers.

Our study showed that both of these facets of the bone metastatic niche were significantly changed in the presence of tungsten. We demonstrated that exposure to tungsten, in the drinking water of mice at a level of 15 ppm, resulted in tungsten accumulation within the bone and several notable changes in the microenvironment within the bone niche including increased osteolysis. Furthermore, we observed changes to the immune response in the bone niche that can drive metastasis including increased numbers of G-MDSCs, activated MSCs, and increase levels of pro-inflammatory/pro-metastatic markers TNFα, IL-1β, Acta-2 and Hif-1a reflective in a pro-tumorigenic inflammatory environment in the bone niche.

However, in the process of trying to identify a distinct mechanism driving these changes, has provided more questions than answers. Therefore, our hypothesis for aim 2*,* “Tungsten will increase the number of osteoclasts in the bone metastatic niche and increase pro-osteolytic cytokines and signals in the bone niche” has yet to be fully answered. We have provided sufficient data to suggest that signaling within the bone niche is affected by tungsten, and shown that direct effects of tungsten on tumor cells may also be contributing to these changes. However, the exact etiology behind the changes to the bone microenvironment following tungsten exposure and enhanced metastasis to the bone remain a point of question of which I will elaborate on in future implications.

It has been reliably shown that tungsten has negative impacts on health especially within the presence of other health stressors. This has not only been demonstrated within our lab but has been supported by multiple other studies. (Keith et al., 2007; EPA 2017) Our data not only adds to this consensus, but shows for the first time that tungsten enhances breast cancer metastasis to the bone, providing evidence that the bone maybe an important site for tungsten-mediated tumorigenesis. This discovery has large health implications and adds to the rapidly accumulating evidence showing tungsten exposure negatively impacts many different populations.

Study Limitations

As mentioned before breast cancer metastasis is a complicated and multifactorial disease state. Therefore, care was taken to isolate variables within our study in an attempt to determine tungsten's role in the disease state. However, I believe a large limitation for this study was being underpowered. Many of my experiments ideally would have had a larger sample size in an attempt to reduce variance. Even though these studies were somewhat underpowered we performed several separate studies in an attempt to reduce variance and demonstrate repeatability. Furthermore, the analysis was rigorously performed as we increased the number of assays and experiments that were performed. By utilizing multiple, repeated measurements from the mice and cell lines, high variability was minimized in the study design.

Another limitation involved challenges to the quantification of changes to the bone microenvironment. One such example of the challenges we had in the quantification of changes to the bone microenvironment revolves around trying to identify the mechanisms underlying the increased osteolysis that was identified following tungsten exposure. We attempted to quantify the number of osteoclasts and

osteoblasts within the bone through TRAP and ALP staining. However, we were unable to replicate results found in previous studies showing a disruption in the bone homeostasis through an increase the number of osteoclasts in the bone following tungsten-exposure *in vivo*. (Chou et al., 2021) For future directions it is possible that by manually counting the number of multinucleated TRAP-positive osteoclasts we may see some degree of change similar to the previously mentioned study. However, it is important to note that the change in osteoclast number was only identified in male mice previously. So, the fact that no change was observed in osteoclast number in our model is consistent with the sex-specific effects observed before. Furthermore, observing the tumor-bearing mice at an earlier point in tumor progression may have identified if osteolysis and increased osteoclasts activity had already occurred. This identification may help us to identify if osteoclast and bone remodeling changes are occurring in a time dependent fashion when exposed to tungsten in breast cancer-exposed animals.

Another self-identified limitation we found ourselves having was having to quantify metastases outside of the bone. Originally tissue sections were stained with H&E and metastasis was evaluated manually using a microscope and visual identification, however we found it difficult to directly quantify metastases within the images. Our expertise in identifying bone metastases was limited and utilizing help of an expert in

histology could have further strengthened our data. Furthermore, while our results show a marked increase in the number of metastases once plated, they do not reflect an accurate number of actual metastases within the bone niche. However, our data might also be telling us that in this model tungsten is driving colonization and growth of tumor cells in the bone and at the time point evaluated only micro metastases had formed. In the future it would be important to potentially extend our studies to evaluate bone metastasis at later time points following tungsten exposure.

Finally, there would have to be substantial evidence to prove the concept of tungsten-driven tumorigenesis and metastasis. Our research has shown significant support for the concept and has showed striking results but, there is still much more research needed to identify a definite mechanism. If the specific effects of tungsten on tumorigenesis are proven to be multifactorial, for example if it were shown to increase osteolysis and tumor recruiting factors, then I believe our research would be able to reliable show and support the growing consensus that oral tungstate exposure is of great environmental concern.

Implications for Future Research

There is still much to be done in determining how tungsten drives breast cancer osteolysis. Of note we attempted to quantify the number of osteoblasts and osteoclasts through ALP and TRAP staining respectively in the bone niche. There is question as to how tungsten affects osteoblast and osteoclast numbers/activity as previous studies have shown an increase in TRAP positive cells, but only in juvenile male mice. Clarification of the mechanisms behind bone osteolysis would help to paint a better picture of how tungsten might affect the bone microenvironment to drive osteolysis. Furthermore, a study focused on the use of bisphosphonates would help determine how suppression of osteoclast activity would affect tungsten accumulation, metastasis, and osteolysis in the bone niche.

We have reliably shown that tungsten increases the number of metastases within the bone marrow, however we do not have quantifiable data from the bone showing metastases. Therefore, several different avenues have been suggested to us for future studies. It has been suggested that resecting the primary tumor may allow for the experiment to continue for longer periods. This would allow bone metastases to grow to a larger size thereby allowing us to better identify metastases within the bone following tungsten exposure. However, the real-world applications of

this study would be hard to quantify as such treatments are often followed by aggressive pharmacologic treatment. Therefore, by sending future bone sections stained for histological examination we may be better able to quantify the number of metastases within the bone. Finally, we have identified a protocol using pan cytokeratin that stains 4T1 positive cells red. It is possible that utilization of this staining in conjunction with histological examination may make identification of metastatic cells in the bone niche more efficient. (Steenbrugge et al., 2019)

Finally, it should be stated that we would like to delineate the direct effects of tungsten on dormant breast cancer cells versus indirect effects of tungsten on the microenvironment in the bone niche to drive metastasis. I have spoken at length about how multifactorial the metastatic process is. Therefore, further dissection of tungsten's role in these processes are important for determining goals and future targets. In an attempt to start this process, we have taken steps to determine the direct molecular mechanisms of tungsten driven changes in 4T1 cancer cells exposed to tungsten *in vitro.* These results from our *in vitro* experiments suggested our *in vivo* data showing increases to genes IL-1β , and especially Acta-2 are the result of tungsten directly acting on the breast cancer cells. It is therefore my opinion that future directions should be aimed at further determining molecular mechanisms within the bone niche following tungsten exposure. Attempts should be aimed at further

differentiating tungsten-mediated changes in populations of cells within the bone niche to determine how osteolysis is occurring in a more linear fashion.

Conclusions

Results from this thesis project showed that mice exposed to oral tungstates, then injected with 4T1 breast cancer cells, exhibit increased breast cancer metastases to the bone. In female BALB/c mice after 4 weeks of oral tungsten exposure. Tungsten enhanced bone metastasis was driven by osteolysis and an induction in a pro-metastatic inflammatory microenvironment in the bone niche. The implications of this work have identified the bone niche as an important site in the pathogenesis of tungsten-enhanced tumor promotion that should be explored further.

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