CHARACTERIZING THE CALCIUM SENSING RECEPTOR IN THE PROGRESSION OF THE METASTATIC PHENOTYPE OF PROSTATE CANCER CELLS

by

Blaine Sherman, BA

A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Human Nutrition
August 2018

Committee Members:

Ramona Salcedo Price, Chair

Krystle Zuniga

Nicholas Bishop

FAIR USE AND AUTHOR'S PERMISSION STATEMENT

Fair Use

Duplication Permission

DEDICATION

I dedicate this work to my family. Thank you for the love, support, and motivation.

ACKNOWLEDGEMENTS

I would like to thank the support of my family. I could not have completed this research without their support. I am grateful for everything that has been provided for me at Texas State University. I have learned so much while working on this project, and I don't think my experience would have been as good at any other institution. I would like to thank my mentor, Dr. Salcedo-Price for all the support and training along the way. I would also like to thank my committee members Dr. Zuniga and Dr. Bishop for being so helpful.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

Abbreviation	Description
Calcium	Calcium
CaMK	Calmodulin Kinase
CaSR	Calcium Sensing Receptor
CCL	C-C Motif Ligand
CCR	C-C Motif Receptor
COX	Cyclooxygenase
CXCL	C-X-C Motif Ligand
CXCR	C-X-C Motif Receptor
DAG	Diacyl Glycerol
EP	E-Prostanoid
IGF-1	Insulin Like Growth Factor-1
IGFBP	Insulin Like Growth Factor Binding Protein
IκBα	Inhibitor of Kappa B Kinases
IL	Interleukin
NF-κB	Nuclear Factor-Kappa B
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5, Diphenyl Tetrazolium Bromide
MMP	Matrix Metallopeptidase
PG	Prostaglandin

PIA ..... Prostatic Inflammatory Atrophy

PIN ..... Prostatic Intraepithelial Neoplasia

PKC..... Protein Kinase C

PTHrP .....Parathyroid Hormone Related Peptide

RANK ..... Receptor Activator of Nuclear Factor Kappa-B

RANKL.....Receptor Activator of Nuclear Factor Kappa-B Ligand

TNF-α..... Tumor Necrosis Factor-alpha

TRP .....Transient Receptor Potential

VEGF ..... Vascular Endothelial Growth Factor

ABSTRACT

Background: Prostate cancer (PCa) is one of the most common cancers in the world. Obesity increases risk for fatal PCa and increases the aggressive phenotype of PCa cells. Calcium sensing receptor (CaSR) is correlated with lethal PCa and bone metastasis which is the most likely site for secondary PCa tumors. *We hypothesize* sera from obese males increase CaSR-mediated PCa progression.

Methods: PC3 and DU145 PCa cells were treated with 5% sera from obese (BMI>30) or normal weight (BMI<25) males. Cells were treated with 5μM 2-Chloro-6-[(2R)-3-[[1,1-dimethyl-2-(naphthalenyl)ethyl]amino-2-hyroxypropoxy]benzonitrile hydrochloride (NPS-2143) to determine the effect of CaSR on PCa cells. Western blot analysis determined protein expression of pAKT, pERK, tumor necrosis factor (TNF)-α, cyclooxygenase (COX-2), interleukin (IL)-6, and CaSR. mRNA levels of ion channels important to PCa progression including receptor activator of nuclear factor kappa-B (RANK), RANKL, CaSR, parathyroid hormone related peptide (PTHrP), transient receptor potential (TRP)M7, and TRPC6 were also assessed. MMP-9 activity, invasive capacity, and wound healing were also assessed.

Results: Obese sera increased the invasive capacity of PCa cells, as well as de-localized EMT markers from the cell membranes which was attenuated with CaSR inhibition. CaSR contributed to MMP-9 activity in DU145 cells treated with normal weight sera. CaSR contributed to mRNA expression of PTHrP in PC3 cells treated with obese sera. Obese sera increased and protein expression of COX-2, IL-6, and CaSR in

DU145 cells. Inhibition of CaSR decreased protein levels of phosphorylated ERK in DU145 cells treated with obese sera, and TNF-α in DU145 cells treated with normal weight sera.

Conclusion: Obese sera was shown to promote the invasive-phenotype and protein expression of important factors in PCa tumorigenesis. The obesity-mediated invasive capacity and expression of COX-2 and IL-6 were similar to control when treated with the CaSR inhibitor, NPS-2143. CaSR may be an important protein to target for obesity-mediated PCa progression.

CHAPTER I

REVIEW OF THE LITERATURE

Introduction

Prostate Cancer

Prostate cancer (PCa) is one of the most common cancers, affecting approximately 3.3 million individuals in the United States.[1] There is evidence that diets high in calcium may increase the risk for PCa, but conclusive evidence thus far does not exist.[2] Mechanisms linking high calcium consumption to PCa include decreased formation of 1,25-dihydroxy vitamin D[3,4] and increased insulin like growth factor (IGF)-1 production associated with high intake of dairy products.[5] There appears to be a U-shaped curve associated with calcium consumption; moderate calcium intake provides the most benefit, while low and high calcium diets increase risk for PCa.[6]

The development of clinical PCa involves several steps including initiation, progression, and advancement.[7] Prostatic inflammatory atrophy (PIA) can be detected in atrophic and highly proliferative cells of the prostate which may progress into prostatic intraepithelial neoplasia (PIN) during initiation.[8] PIN may progress into invasive carcinoma when the basal cells and basement membrane are depleted. Neoplasia may undergo further advancement into aggressive, metastatic, invasive, or castration resistant phenotype.[8] Treatment options for PCa include active surveillance, hormone therapy,

radiation therapy, surgery, chemotherapy, cryotherapy, vaccine treatment, and bone directed treatment.[1,8,9] Hormone therapy employs the deprivation of growth signaling androgens such as testosterone and 5-α dihydrotestosterone via chemical or surgical castration.[8] The removal of androgens inhibits growth of PCa cells, but over time, resistance to this therapy may occur. PCa that is resistant to therapy and develops an invasive phenotype is most likely to metastasize to the bone.[10] Cells that migrate beyond the primary tumor site secrete factors that communicate and attract them to the bone. The bone also secretes factors that are received by the PCa cells which can lead to the development of a secondary tumor site in the bone.

Obesity and Prostate Cancer

Obesity has been identified as a risk factor for advanced prostate cancer.[8,11] A meta-analysis shows that an increase of BMI by 5 kg/m^2 in undiagnosed males is associated with a 15% increased risk for future mortality caused by PCa.[12] Individuals with previously diagnosed prostate cancer had a 21% increased risk for recurrence associated with BMI increase of 5 kg/m^2.[12] Obesity is linked to the hallmarks of cancer through the sustained growth signaling, initiation of invasion and metastasis, stimulation of angiogenesis, production of tumor promoting inflammatory factors, decrease of genetic stability, evasion of immune destruction, and dysregulating cell energetics.[13,14] Mechanisms that have been linked to obesity and tumor promoting inflammation include the dysregulation of cell signaling, alterations to the microenvironment, systemic inflammation, and disruption of the vasculature.[15] Obesity is a state of chronic inflammation resulting in increased production of inflammatory cytokines including IL-1β, IL-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1).[14–17] Obesity-related

inflammatory cytokines in conjunction with increased free IGF-1 and leptin result in the activation of nuclear factor-kappa B (NF-kB), an important transcription factor in PCa tumorigenesis.[18,19] IGF-1 promotes the activation of the PI3/AKT and Ras/MAPK pathways which are associated with tumorigenesis.[20] Obesity causes increased production of the hormone leptin, which causes cell signal dysregulation, immune system dysfunction, and angiogenesis.[15,21,22]

Obesity causes alterations to the various microenvironments PCa cells are exposed to during the development of metastasis. Increased angiogenesis is required for the growth of adipose tissue and promotes the delivery of oxygen and nutrients to PCa cells. Angiogenesis is dependent on factors such as vascular endothelial growth factor (VEGF), IGF-1, and leptin which contribute to tumorigenesis.[23] Another critical component of the microenvironment includes the periprostatic adipose tissue in which genes and protein expression are altered by PCa cells and obesity-related cytokines.[17] Treating primary periprostatic tissue with conditioned media from PCa cells results in increased matrix metallopeptidase (MMP)-9, mitochondrial DNA copy number, osteopontin, TNF-α, and decreased adiponectin.[17] These changes alter the tumor microenvironment, increase proliferation, and promote the invasive phenotype of the PCa cells.[17] The bone microenvironment includes growth factors and chemo-attractants that attract PCa cells to the bone.[24] Migratory PCa cells in proximity to bone cause increased bone remodeling through the exchange of cell signals. Bone remodeling results in increased levels of these growth factors and chemo-attractants including transforming growth factor (TGF)-β, IGF-1, and PTHrP.[24–26] Obesity contributes to the invasive

phenotype of PCa by increasing proliferation, migration, MMP activity, and stimulating angiogenesis.

Nuclear Factor Kappa-B (NF-κB)

NF-κB transcription factors are important contributors to the inflammatory environment and provide a link between inflammation and cancer.[18] NF-κB is activated by inflammatory factors including IL-1 and TNF-α.[27] The response of NF-κB to inflammatory stimulation is to increase the gene expression of pro-inflammatory genes including cytokines, adhesion molecules, and chemokines.[27] NF-κB activation also increases the expression of tumorigenic genes that control cell-cycle progression, angiogenesis, and resistance to apoptosis.[18] CaSR expression has been shown to increase in response to IL-1β and TNF-α following NF-κB activation in kidney, thyroid, and parathyroid.[28] This research indicates CaSR modulates NF-κB in response to stress leading to alterations in calcium homeostasis in various tissues. NF-κB is activated in response to inflammatory factors that are elevated in the obese state, and leads to gene transcription for factors that are important to the invasive phenotype of PC.

NF-κB is found in its inactive state in the cytosol bound to IκB.[29] In order for NF-κB to carry out its function the IκB must be degraded, releasing NF-κB so it can be localized to the nucleus and carry out its function.[29] This activation takes part upstream of the NF-κB/IκB complex upon activation of IκB kinase complexes, which are typically composed of IKKβ and IKKα subunits.[29] The N-terminal of IκB proteins are responsive to the stimulation of phosphorylation from upstream IKK subunits. The resulting phosphorylation causes the IκB protein to undergo ubiquitination, then proteasomal

degradation.[30] The degradation of IκBα depends on the phosphorylation and thus activation of the serine units S32 and S36.[29] TNF-α and IL-1 are both increased with obesity, important mediators of inflammation, and known to degrade IκBα leading to the activation of NF-κB.[29]

Ras/Raf/MEK/ERK Signaling Pathway

The Ras/Raf/MEK/ERK signaling pathway has been shown to be upregulated in many cases of advanced PCa and is associated with poor prognosis.[31] Mitogens and growth factors trigger the Ras/Raf/MEK/ERK signaling pathway to regulate cell cycle, apoptosis, and gene expression.[31] In the cytosol, ERK can phosphorylate molecules that regulate apoptosis and in the nucleus, ERK can phosphorylate and activate c-Myc, CREB, c-Jun, c-Fos, and Ets leading to the transcription of genes that regulate proliferation and prevent apoptosis.[31] Reactive oxygen species (ROS) are also activators of Raf/MEK/ERK signaling cascade, and ROS have been shown to be upregulated in PCa cells and is increased with obsesity.[31–33]

Interleukin-6 (IL-6)

Obesity causes increased levels of IL-6 which is associated with both resistance to treatment and advanced metastatic PCa.[34] IL-6 contributes to the generation of osteoclast which result in increased bone remodeling, and promotes osteoblast differentiation through the JAK/STAT pathway.[35] IL-6 has also been shown to increase MMP-9 activity through PI3K/AKT signaling.[36] Long term treatment of IL-6 in LNCaP cells increased VEGF which was dependent on PI3K and increases the invasive capacity.[37] IL-6 treatment has been shown to increase several contributors of EMT regulated through

cancer associated fibroblasts that also secreted MMP-9 in an IL-6 dependent manner.[38] This shows the importance of IL-6 in the tumor microenvironment leading to increased migratory capacity. IL-6 is important for the progression to castration resistant PCa by activating the androgen receptor, increasing androgen receptor gene expression, and promoting growth in low androgen conditions.[39,40] IL-6 is upregulated in obese individuals and causes the development of an invasive phenotype in PCa by activating various transcription factors that are important to the inflammatory response including NF-κB and JAK/STAT pathways. IL-6 is also an important contributor to bone remodeling which causes the release of growth factors and calcium from the bone leading to increased PTHrP excretion in PCa cells.

Tumor Necrosis Factor-alpha (TNF-α)

TNF-α is an important cytokine in the progression of obesity-mediated PCa, despite its name as a tumor necrosis factor. Elevated levels of TNF-α is common in obese individuals, and its secretion is upregulated by proinflammatory M1 macrophages in adipocytes.[41] TNF-α activates both NF-κB and JNK, and increases risk for EMT and DNA damage.[41] Conditioned media from PCa cells increase the production of TNF-α in adipocytes leading to increased cell growth and the invasive phenotype.[17] The addition of TNF-α to LNCaP cells led to increased expression of genes important to PCa progression including COX-2, MMP-9, VEGF, and IL-6.[19] In each instance, blocking PKCε with the inhibitor εV1-2 decreased the expression of the gene.[19] Each gene also had increased expression in comparison to control when PKCε was overexpressed.[19] PKCε translocation to the membrane was required and led to IKK phosphorylation and NF-kB

activation.[19] TNF-α is upregulated in obese individuals and contributes to the inflammatory response that has been shown to increase the invasive capacity of PCa.

Insulin-like Growth Factor-1 (IGF-1)

IGF-1 is a growth factor found throughout the body that has mitogenic, angiogenic, and metastatic effects in PCa.[42] IGF-1 has several receptors that are dysregulated in PCa among other cancers and obesity.[42,43] Downstream effects of IGF-1 activity include phosphorylation of ERK leading to increased proliferation, and phosphorylation of AKT leading to increased protein synthesis, decreased apoptosis, and increased glucose metabolism.[44] Obesity causes increased levels of free IGF-1 due to increased synthesis of IGF-1 and decreased synthesis of IGF-1 binding proteins.[43] PCa cells expressing CaSR release PTHrP which stimulates osteoclast precursors. Mature osteoclast precursors cause increased bone remodeling which results in the release of IGF-1, and calcium from the bone environment.[43,45] The increased concentration of calcium stimulates the release of more PTHrP, and IGF-1 promotes the growth of the cancerous cells.[24,46] This cycle continues, and the IGF-1 among other factors attract the PCa cells to the bone.[24,45] IGF-1 levels are dysregulated in obesity, and contributes to the invasive phenotype of PCa by increasing proliferation, angiogenesis, migratory behavior, and contributes to bone remodeling. Diets high in minerals including calcium have been correlated to increased levels of IGF-1 and IGF-1:IGFBP-3.[5] This could be part of the contribution of CaSR to the inflammatory environment associated with PCa and obesity.

Cyclooxygenase-2

COX-2 is an inducible enzyme that converts arachidonic acid to prostaglandin (PG)-H2, which is the precursor for physiologically active PGs including PGE2.[47,48] PGHS-2 is induced in response to inflammation reacting with factors such as IL-1 and TNF-α.[47] Constitutive or inducible activation of PGHS-2 occurs in several types of tissues and biological processes such as bone turnover.[47] TNF-α and IL-1β share common inflammation-mediated pathways that regulate the transcription of PGHS-2 including NF-kB, C/EBP, and MAPK signaling cascades that are important for the development of invasive PCa.[47] The COX-2 inhibitors celecoxib and CAY10404 decreased proliferation in a dose dependent manner in the PCa cell lines LNCaP, PC3, and 22RV1.[48] The EP (E-Prostanoid)-1 receptor is activated by its ligand PGE2,[49] and was shown to be required for proliferation in LNCaP cells.[48] EP1 was previously shown to exert its downstream actions by coupling to the G-proteins Gαq and Gα(i/o) leading to calcium influx and activation of PKC and AKT.[48–50] COX-2 is an important mediator of obesity-induced inflammation, and has been identified as an important enzyme in PCa tumorigenesis.

CaSR and PCa Progression

CaSR is a G-coupled protein that is highly sensitive to extracellular calcium and plays an important role in calcium homeostasis.[25] The extracellular protein is important for the function of various organs such as the parathyroid and the kidney.[25] CaSR has also been shown to be important in the progression of several diseases including various cancers.[25] Primary PCa tumors with increased expression of CaSR have been linked to increased risk for bone metastasis.[6] CaSR is expressed in both osteoblast and osteoclast,

and has been linked to cross-talk between cancer cells and osteocytes.[24,51] In PCa cells, CaSR promotes the excretion of parathyroid hormone related peptide (PTHrP) which causes cyclic increases in both bone remodeling and PTHrP excretion. It has been proposed CaSR in healthy cells prevents the release for PTHrP through the inhibition of the CaSR subunit Gαi, causing the inhibition of cAMP production.[24,52] Cancerous cells can activate the CaSR subunit Gαs, resulting in increased cAMP and PTHrP excretion.[18,53] PTHrP increases bone remodeling and causes the release of calcium and growth factors from the bone. This causes a further increase of PTHrP production from cancerous cells, thus increasing osteoclastogenesis and bone remodeling.[24,52,53]

CaSR has been shown to be responsive to inflammatory factors in other organs including the parathyroid, kidney, and adipose tissue. In an *in vivo* model, injection of IL-6 increased CaSR expression in the parathyroid and kidney.[54] Differentiated LS14 adipocytes treated with IL-6, IL-1β, or TNF-α resulted in increased levels of CaSR protein.[55] The adipocytes also increased CaSR expression following treatment from conditioned medium from the adipose tissue of individuals with high BMIs.[55] CaSR has been identified to play an important role in the inflammatory response from various tissues, however there is a *gap of knowledge* in the inflammatory response in PCa cells.

Ion Channels and PCa Progression

Alterations in calcium influx has been identified as a major player in the progression of PCa. Calcium is important for cell function, and maintaining intracellular and extracellular calcium concentrations is critical for homeostasis.[56,57] Calcium ion channels TRPM7, ORAI1/ORAI3, TRPV6, and TRPC6 have been associated with

proliferation of PCa cells through pathways including ERK, calcineurin A, calmodulin kinase II (CaMK), and NFAT (Table 1).[56] The ion channels TRPM7, TRPV2, and TRPM8 have been associated with the increased metastatic and invasiveness of PCa cells through alterations of e-cadherin via calpain, and focal adhesion degradation via phosphorylation of focal adhesion kinases bound to integrins (Table 1).[56] Ion channels such as TRPC6 have been shown to be influenced by CaSR and it is important to identify the link between other ion channels with obesity, PCa tumorigenesis, and CaSR expression. It is important to elucidate which ion channels are affected by obesity, and these channels effect on calcium homeostasis and cell signaling, could be an important mediator in obesity-related invasive PCa.

Table 1: Characteristics of Calcium Ion Channels Associated with PCa. The ion channels ORAI1 and ORAI3 have been associated with either calcium homeostasis or loss of homeostasis and tumorigenesis. Several calcium ion channels have been associated with increased proliferation including TRPM7, TRPC6, and TPRV6. Ion channels that have been associated with migration including TRPM7, TRPV2, and TRPM8.

Ion Channel	Role in PCa
ORAI1	↓ Risk for tumorigenesis[58]
ORAI3	↓ Function of ORAI1[58] ↓ Calcium Homeostasis Dysregulate cell signaling[58] Arachidonic acid regulation of calcium influx[58] ↑ COX-2[58]
TRPM7	↑ Proliferation[59] Mg: Ca2+ ratio[59] Calcium influx[59] Activation via cholesterol[59] Phosphorylation of AKT and ERK[59] ↑ Metastasis[60] ↓ e-cadherin[60]
TRPV6	Associated with Gleason score >7[68] ↑ Proliferation[69] ↑ Calcium influx[61]
TRPC6	Increased expression with high calcium diet[62] ↑ Proliferation[62]
TRPV2	↑ Migration ↑ MMP-9[63] ↑ MMP-2[63] 15-fold higher expression in metastatic tumors[63] Associated with androgen independence[63]
TRPM8	↓ Migration ↓ VEGF

Phospholipase C (PLC)

Growth factors and hormones activate phospholipase C by activating receptor tyrosine kinases (RTCs) or G-protein coupled receptors.[64] Upon activation PLC hydrolyze phosphoinositides resulting in increased intracellular calcium, which is released from the endoplasmic reticulum and increase diacyl glycerol (DAG).[64] Both calcium and DAG have many cell-signaling functions within a cell. The hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) results in IP3 and DAG which are intracellular second messengers.[65] IP3 binds to its receptor IP3R causing calcium release from the endoplasmic reticulum. Increased levels of DAG results in the activation of PKC resulting in changes to the regulation of many cellular pathways including that of calcium signaling.[65] The primary intracellular calcium sensors include CaMK and calcineurin.[65] CaMKs have been shown to phosphorylate proteins important to inflammatory responses including JNK and p38, and activate transcription factors such as FOXO and CREB.[65]

Conclusion

Obesity contributes to the invasive phenotype of PCa through several mechanisms, including inflammation which increases the risk for metastasis and mortality. Increased expression of CaSR is correlated with bone metastasis and plays a role in cyclic progression of tumor invasion into skeletal formations. CaSR has been shown to contribute to a pro-inflammatory environment and respond to inflammation in several tissues. By increasing our understanding of cross-talk of these mediators of PCa progression we can elucidate targets for therapeutic intervention that could lead to

decreased prevalence, shorter time-frame of therapy, decreased complications, and longer survival rates. Obesity may alter several factors leading to aberrant calcium signaling and influx including TRPM7, TRPC6, TRPV6, TRPV2, TRPM8, ORAI1, and ORAI3. Obesity-mediated inflammation may play a pivotal role in increasing secretion of PTHrP leading to a cycle of increased PCa cell growth and increased bone remodeling. Our aim is to determine the contribution of CaSR to obesity-mediated PCa progression in an *in vitro* model using PCa cells and the CaSR inhibitor NPS-2143.

Objectives

This study addresses the contribution of CaSR to obesity-mediated cell signaling, inflammation, and invasive capacity in PCa cells. An *in vitro* model was used to treat androgen independent PCa cells, PC3 and DU145, with pooled sera derived from males that are obese or normal weight. PCa cells were treated with or without the CaSR inhibitor NPS-2143 to assess the contribution of CaSR in obesity-mediated aggressive phenotype of PCa cells. To determine if obesity upregulates CaSR, cells were treated with obese and normal weight sera and CaSR protein expression was measured. To determine the contribution of obese sera, normal weight sera, and CaSR to cell viability cells were treated with sera and the CaSR inhibitor and cell viability was measured. The invasive phenotype was measured following treatment with obese sera and NPS-2143. Localization of β-catenin and e-cadherin were visualized in a qualitative manner to visualize markers of EMT, which is important for the generation of secondary tumors. Contributors to cell signaling and tumorigenesis were assessed by measuring activation of ERK and AKT. The inflammatory mediators TNF-α, COX-2, and IL-6 were measured by western blot analysis. PTHrP mRNA was measured to determine if obese sera and

CaSR upregulate PTHrP which would increase bone remodeling and risk for bone metastasis. mRNA for TRPC6 and TRPM7 were measured to determine if obese sera and CaSR increase the ion channel mRNA which is important for PCa tumorigenesis and intracellular calcium homeostasis. mRNA for RANK and RANKL were determined to assess the contribution of obese sera and CaSR for the two molecules that are important for the generation of bone metastasis.

CHAPTER II

OBESITY, PROSTATE CANCER, AND CALCIUM SENSING RECEPTOR

Abstract

Background: Prostate cancer (PCa) is one of the most common cancers in the world. Obesity increases risk for fatal PCa and increases the aggressive phenotype of PCa cells. Calcium sensing receptor (CaSR) is correlated with lethal PCa and bone metastasis which is the most likely site for secondary PCa tumors. *We hypothesize* sera from obese males increase CaSR-mediated PCa progression.

Methods: PC3 and DU145 PCa cells were treated with 5% sera from obese (BMI>30) or normal weight (BMI<25) males. 5μM NPS-2143 was used to determine the effect of CaSR on PCa cells. Western blot analysis was used to determine protein expression of pAKT, pERK, tumor necrosis factor (TNF)-α, cyclooxygenase (COX-2), interleukin (IL)-6, and CaSR. qRT-PCR was used to determine mRNA levels of ion channels important to PCa progression including receptor activator of nuclear factor kappa-B (RANK), RANKL, CaSR, and parathyroid hormone related peptide (PTHrP), transient receptor potential (TRP)M7, and TRPC6. Zymography was used to determine differences in MMP-9 activity, Matrigel Invasion Chambers were used to determine invasive capacity, and wound healing assay was used to determine motility of PCa cells.

Results: Obese sera increased the invasive capacity of PCa cells, as well as de-localized EMT markers from the cell membranes which was attenuated with CaSR

inhibition. CaSR contributed to MMP-9 activity in DU145 cells treated with normal weight sera. CaSR contributed to mRNA expression of PTHrP in PC3 cells treated with obese sera. Obese sera increased and protein expression of COX-2, IL-6, and CaSR in DU145 cells. Inhibition of CaSR decreased protein levels of phosphorylated ERK in DU145 cells treated with obese sera, and TNF-α in DU145 cells treated with normal weight sera.

Conclusion: Obese sera was shown to promote the invasive-phenotype and protein expression of important factors in PCa tumorigenesis including CaSR, COX-2, and IL-6 in DU145 cells. The obesity-mediated invasive capacity and expression of several inflammatory mediators including COX-2 and IL-6 in DU145 cells were similar to control when treated with the CaSR inhibitor, NPS-2143. CaSR may be an important protein to target for obesity-mediated PCa progression.

Introduction

Obesity has been established as a risk factor of aggressive forms of PCa resulting in complications with diagnosis, treatment, and increased mortality.[11,66,67] The mechanisms linking obesity to aggressive PCa have not been fully understood although inflammation, alterations in hormone signaling, and cytokine signaling play significant roles in obesity-related PCa. Primary PCa tumors often metastasize to the bone, and obesity increases bone turnover which increases the risk for bone metastasis. Obesity is linked to the hallmarks of cancer through the sustained growth signaling, initiation of invasion and metastasis, stimulation of angiogenesis, production of tumor promoting inflammatory factors, decrease of genetic stability, evasion of immune destruction, and dysregulation of cell energetics.[13,14] Obesity is a state of chronic inflammation, resulting in increased production of inflammatory cytokines important to PCa aggressiveness including IL-1β, IL-6, and TNF-α.[14–17]

Obesity causes alterations to the various microenvironments PCa cells are exposed to including increased free IGF-1, leptin, PTHrP, TGF-β, MMP-9, TNF-α, IL-6, IL-1β, and VEGF.[17,23–26] Increased levels of IL-6 lead to androgen independence and increased MMP-9, VEGF, and markers of EMT.[37–40] Obesity-mediated inflammatory cytokines include TNF-α, which increases COX-2, MMP-9, VEGF, IL-6, DNA damage, and risk for EMT.[19,41] COX-2 is an inducible enzyme that converts arachidonic acid to PG-H2, the precursor for physiologically active PGs including PGE2, which is important for tumorigenesis.[47,48]

There is evidence that diets high in calcium may increase the risk for PCa, but conclusive evidence thus far does not exist.[2] There appears to be a U-shaped curve associated with calcium consumption; moderate calcium intake provides the most benefit, while low and high calcium diets increase risk for PCa.[6] High calcium diets in a murine model, led to increased gene expression of both CaSR and TRPC6.[62] CaSR has been associated with inflammation and the hallmarks of cancer with both inhibitory and promoting effects depending on the type of cancer.[68] Elevated levels of CaSR have been associated with increased risk for deadly forms of PCa, and metastasis to the bone.[69,70]

CaSR is a G-coupled protein that is highly sensitive to extracellular calcium, and plays an important role in calcium homeostasis.[25] PCa tumors with increased expression of CaSR have been linked to increased risk for bone metastasis.[6] CaSR is expressed in both osteoblast and osteoclast, and has been linked to cross-talk between cancer cells and osteocytes.[24,51] In PCa cells, CaSR promotes the excretion of PTHrP which causes cyclic increases in both bone remodeling and PTHrP excretion. It has been proposed CaSR in healthy cells prevents the release for PTHrP, through the inhibition of the CaSR subunit Gαi resulting in the inhibition of cAMP production.[24,52] In cancerous cells, CaSR can increase activation of the subunit Gαs resulting in increased cAMP and PTHrP excretion.[18,53] PTHrP increases bone remodeling and causes the release of calcium and growth factors from the bone. Increased calcium and growth factors results in a further increase of PTHrP production from cancerous cells, thus increasing osteoclastogenesis and bone remodeling.[24,52,53]

CaSR has been shown to be upregulated following exposure to obesity-mediated inflammatory factors in the parathyroid, thyroid, kidney, and differentiated

adipocytes.[28,54,55] Differentiated adipocytes also increased CaSR expression following treatment from conditioned medium from the adipose tissue of individuals with high BMIs.[55] CaSR has been identified to play an important role in the inflammatory response from various tissues, however there is a *gap of knowledge* in the inflammatory response in PCa cells.

Alterations in calcium influx have been identified as an important factor in the progression of PCa. Calcium ion channels TRPM7, ORAI1/ORAI3, TRPV6, and TRPC6 have been associated with proliferation of PCa cells through pathways including ERK, calcineurin A, CaMK, and NFAT.[56] The ion channels TRPM7, TRPV2, and TRPM8 have been associated with the increased metastatic and invasiveness of PCa cells through alterations of e-cadherin, and focal adhesion degradation.[56] Ion channels such as TRPC6 have been shown to be influenced by CaSR and it is important to identify the link between other ion channels with obesity, PCa tumorigenesis, and CaSR expression.[62]

Our lab has previously shown changes in proliferation, inflammation, invasion, and the EMT in an *in vitro* and *in vivo* model of obesity and PCa.[71,72] For this study the CaSR inhibitor NPS-2143 (5μM) was used to infer differences in the function of CaSR between PCa cells treated with obese or normal weight sera (5%). We measured the contribution of OB and NW sera and CaSR in cell signaling by measuring activation of AKT and ERK, expression of inflammatory mediators including TNF-α, COX-2, and IL-6. PTHrP, TRPC6, TRPM7, RANK, and RANKL mRNA was measured, and the invasive capacity and phenotype was also measured.

Methods

Cell Culture

Human prostate carcinoma cells PC3 and DU145 were purchased from American Culture Collection (ATCC) (Rockville, MD). The PC3 cell line is derived from a bone metastasis, and cells were maintained in F-K12 medium supplemented with 10% fetal bovine serum (FBS). DU145 cells are derived from brain metastasis, and were maintained in MEM with 10% FBS. For experimental procedures, serum starving was done with S-MEM which is free of calcium. All cells were maintained and treatments carried out in an incubator at 37°C and 5%CO_2. To inhibit CaSR, 5μM NPS-2143 was added to cells during serum starving and treatments. All treatments were carried out at three times.

Characterization of Sera

Male sera were obtained from Equitech Enterprises Inc. (Kerrville, TX). Male human sera from body mass index (BMI) category of obese (OB) BMI>30 kg/m^2 (n=5) and normal weight (NW) BMI <25 kg/m^2 (n=5) were pooled and used for *in vitro* studies, and 5% sera was used for treatment conditions. Sera adipokines were measured using an adipokine array assay, (R&D Systems, Minneapolis, MN) and results were previously reported.[72] Calcium concentration of obese sera, normal weight sera, and FBS were characterized using calcium colorimetric assay (Abcam, Cambridge, MA).

Cell Viability Assay

Cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT) reagent following treatment of 5% OB or NW sera with or without the CaSR inhibitor (5μM NPS-2143) for 72 hours. Cells were seeded at 5.0 X 10^4 cells per well in 96-well plates, n=6. Following treatments, 20μL of MTT (5 mg/ml) were added to cells, and formazan salts were formed from active mitochondria indicating viable cells. Formazan salts were solubilized with 100 μL DMSO and absorbance was read at 570 nm wavelength with Cytation™5 Cell Imaging Multi-Mode Reader (Bio Tek Instruments, Inc., Winooski, VT).

Wound Healing Assay

PC3 and DU145 cells were seeded at 4.0 X 10^5 and allowed to grow to 95% confluency. Cells were washed with PBS and serum starved in S-MEM (1.8mM calcium) for 1 hour. A scratch line was made down the confluent monolayer using a 200 μL pipet tip. S-MEM media was replaced with MEM media (1.8mM calcium) and treatments of 1% OB sera and 1% NW sera with or without the addition of 5μM NPS-2143. The reduced concentration of sera was used to reduce the growth of cells, so the assay would be able to measure motility as opposed to growth. Changes in cell migration were quantified using Cytation5. Margins of the scratch were measured at baseline and 24 hours. The margins were measured in three different locations to obtain the size of the wound at each time point. Change in wound closure was calculated as percent wound closure.

Zymography

Differences in proteolytic activity of MMP-9 following exposure to sera and NPS-2143 was assessed with zymography. Cells were seeded at 4.0 X 10^5 cells per well in 6-well plates and allowed to grow to confluency. Cells were then washed with phosphate buffered saline (PBS) and serum starved for 4 hours in S-MEM +/- 5μM NPS-2143. Treatments of 5% OB or NW sera with or without 5μM NPS-2143 were then added for 1 hour. Cells were washed with PBS and serum free media was added for 16-hours. Conditioned media concentrated 14-fold was ran down Ready Gel Zymogram Precast Gels (Bio-Rad Hercules, CA) at 125V for 1.5 hrs. Gels were renatured in 1X Zymogram Renaturing Buffer (Novex Carlsbad, CA) for 30min and developed in 1X Zymogram Developing Buffer (Novex Carlsbad, CA) for 16-hours at 37°C. The following day, gels were stained with Coomassie blue (0.5%) and de-stained with 50 methanol: 20 acetic acid: 30 ddH_20. Gels were scanned at 1200 dots per inch (DPI), then pixilation was measured with ImageJ and standardized to NW sera.

Invasion Assay

Physiological changes in invasive capacity was measured using Corning® BioCoat™ Matrigel® Invasion Chambers (Discovery Labware, Bedford, MA). Invasion chambers were seeded with 5.0 X 10^4 cells per well in 24-well plates, and allowed to attach for 16-hours. The following day, cells were washed with PBS, then exposed to treatments of 5% OB or 5% NW sera with or without 5μM NPS-2143. Media supplemented with 10% FBS was added to the well as a chemoattractant. After a period of 24 hours, cells that migrated through the Matrigel membrane were fixed with 10%

formalin, and stained with 1% crystal violet dissolved in methanol. Images were taken using manual imager mode with Cytation5 imaging reader, and cells were manually counted using one random image.

Immunofluorescence

To visualize differences in EMT proteins immunofluorescence was used, 2.0 X 10^4 cells were seeded for 16-hours on Culture Slides 4-Chamber Polystyrene Vessel Tissue Culture Treated Glass Slide (Falcon Big Flats, NY). The following day, cells were washed with PBS and treated for 16-hours with 5% OB or NW sera with or without 5μM NPS-2143. The following day cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X100. Cells were then stained with 1:50 e-cadherin primary anti-body and 1:100 β-catenin primary antibody. Following primary antibody staining, cells were stained with 1:133 fit-c and cy-3. Cells were also stained with 1:100 Hoechst to detect the nucleus. Representative images were obtained using Cytation5 plate reader.

Quantitative Real-Time PCR (qRT-PCR)

Cells were seeded in 6-well plates at a cell density of 4.0 X 10^5 for 16 hours. Cells were then washed with PBS, then serum starved in S-MEM with or without 5μM NPS-2143 for 1 hour. Cells were then treated with 5% OB or NW sera with or without 5μM NPS-2143 for 4 hours. Following treatment, RNA was extracted using Trizol and cDNA was synthesized using Super Script II according to manufacturer's protocol (Invitrogen, Carlsbad, CA). Primer sequences are identified in Table 2. Protocol for SYBR Select Master Mix (Applied Biosystems Austin, TX) was followed.

Table 2: Table of Primers Used for qRT-PCR.

CaSR	Sense 5'-AAGAAAGTTGAGGCGTGGCAG Antisense 5'-GAGGTCCCAGTTGATGATGGA
PTHrP	Sense 5' to 3': CGGTGTTCCTGCTGAGCTA Antisense 5' to 3': TGCGATCAGATGGTGAAGGA
TRPM7	Sense 5' to 3': CTTATGAAGAGGCAGGTCATGG Antisense 5′ to 3': CATCTTGTCTGAAGGACTG
TRPC6	Sense 5' to 3': TTCCCGCCATGAGCCAC Antisense 5' to 3': CGGTGAGCCAGTCTGTTGTCAGAT
RANK	Sense 5' to 3': CAGGGATCGATCGGTACAGT Antisense 5' to 3': GTTTGAGACCAGGCTGGGTA
RANKL	Sense 5' to 3': GCTTGAAGCTCAGCCTTTTGCTCAT Antisense 5' to 3': GGGGTTGGAGACCTCGATGCTGATT
Actin	Sense 5' to 3': GACCTCTATGCCAACACAGT Antisense 5' to 3': AGTACTTGCGCTCAGGAGGA

Western Blot Analysis

Western blot analysis was used to determine differences in protein expression following exposure to treatment conditions. Cells were seeded at 4.0 X 10^5 cells per well in 6-well plates and allowed to attach for 16-hours. Cells were then washed with PBS, and serum starved in S-MEM with or without 5μM NPS-2143 for 4 hours. To detect changes in phosphorylation of AKT and ERK cells were treated with 5% OB or NW sera with or without the addition of 5μM NPS-2143 for 15 minutes. To detect differences in TNF-α, COX-2, IL-6, and CaSR cells were treated with the same conditions for 4 hours. Following exposure to treatment conditions, cells were washed with ice cold PBS then ice-cold lysis buffer was added. Lysates were harvested and stored at -20°C until proteins were ran down SDS-PAGE gels. Protein concentration was determined using Pierce™ BCA (Bicinchoninic Acid) Protein Assay Kit (Thermo Scientific, Rockford, IL). Proteins were separated using electrophoresis with 10% SDS-PAGE resolving gels, and proteins

were separated at 100V for 1.75hrs. Proteins were transferred to nitrocellulose membranes for 1 hour at 100V, then proteins were blocked with 5% non-fat dry milk in TBST. Membranes were exposed to primary antibodies for phosphorylated-Akt Ser 473 (rabbit polyclonal antibody, Cell Signaling Technology), total Akt (rabbit monoclonal antibody, Cell Signaling Technology), phospho-p44/42 MAPK (ERK1/2) (rabbit monoclonal antibody, Cell Signaling Technology), and p44/42 MAPK (pERK1/2) (rabbit polyclonal antibody, Cell Signaling Technology), CaSR (HL 1499) (mouse monoclonal antibody, Novus Biologicals, Littleton, CO), TNF-α (NBP-19532) (rabbit polyclonal antibody, Novus Biologicals, Littleton, CO), IL-6 (rabbit polyclonal antibody, EMD Millipore Corporation, Temecula CA), and COX-2 (rabbit polyclonal antibody, Thermo Fisher, Rockford, IL) . Membranes were then exposed to corresponding secondary antibody, either mouse or rabbit, followed by exposure to Super Signal™ West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Images were taken using Fotodyne image reader (Harland, WI). Pixel density was measured using ImageJ software (National Institutes of Health). Relative pixilation was expressed as a ratio of protein of interest to actin, and standardized to NW sera.

Statistical Analysis

Values are presented as mean ± standard error of the mean. Experiments were conducted in triplicate. Statistical analysis was performed between treatment groups and the normal weight treatment group, which was used as control. Graph Pad Software (La Jolla, CA) was used for ANOVA, followed by Tukey's multiple comparisons test and *P* value less than 0.05 was considered statistically significant. Data were examined for

extreme values, which were defined as values 3 standard deviations outside the mean (Peirce's criterion); extreme values were excluded from analysis.

Results

Calcium Levels are Similar Between Sera

CaSR is highly sensitive to small differences in extracellular calcium. Therefore, calcium colorimetric assay (Abcam) was used to determine differences between OB and NW sera to ensure calcium concentrations are similar. Calcium concentration of FBS was also identified because it is used to maintain the cells, and as a chemoattractant. Calcium colorimetric assay (figure 1) indicated the calcium levels were similar between OB sera, NW sera, and FBS.

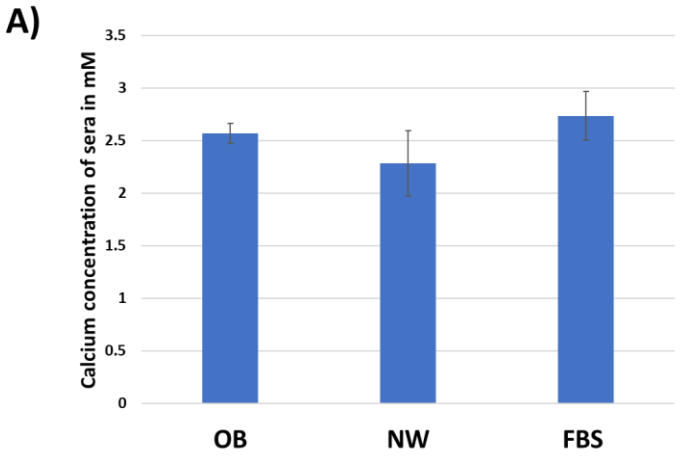


Figure 1. Calcium Concentration of Sera. A) Calcium concentrations were similar between sera types. Obese (OB), normal weight (NW), and fetal bovine sera (FBS). OB and NW sera were pooled from 5 males in each BMI category (OB > 30 kg/m^2, NW < 25 kg/m^2).

Effect of CaSR on Cell Viability

Increased cell viability enables tumor growth, increasing the risk for evasion from the primary microenvironment. To determine the effect of CaSR and OB sera on cell viability we used MTT assay following treatment with OB and NW sera with or without NPS-2143. There were no statistically significant differences between treatment groups (figure 2). This indicates OB sera and CaSR did not contribute to cell viability following exposure for 72 hours.

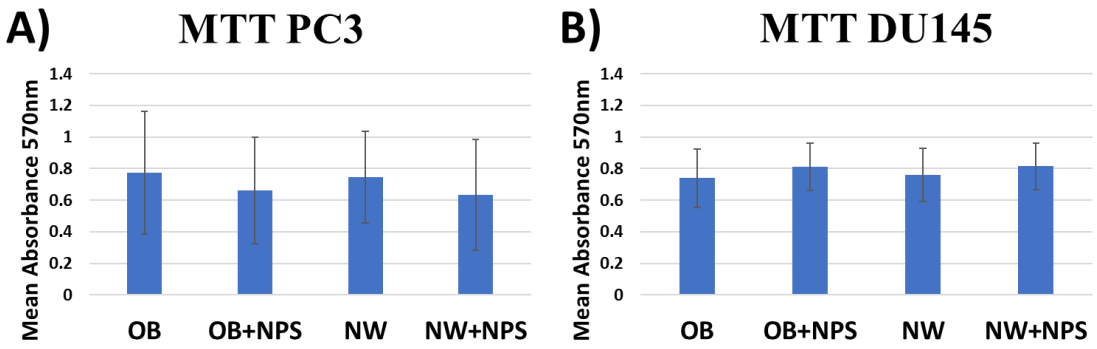


Figure 2. Cell Viability in Response to Human Sera and CaSR Inhibitor. A) PC3 and B) DU145 cells were exposed obese sera (OB), OB with NPS-2143 (OB+2143), normal weight sera (NW), or NW with NPS-2143 (NW+NPS) for 72 h. Cell viability was assessed using MTT dye conversion.

Wound Healing Assay

To determine effects of CaSR and obese sera on cell motility, a wound healing assay was performed. Cells were exposed to OB (1%) or NW (1%) sera with or without 5μM NPS-2143 for 24 hours. The reduced percentage of sera was used to minimize cell growth. PC3 cells had 100% wound closure in all experimental conditions (figure 3). There were no statistically significant differences in wound closure for DU145 cells. This

indicates CaSR does not contribute to obesity-mediated cell motility in PC3 or DU145 cells.

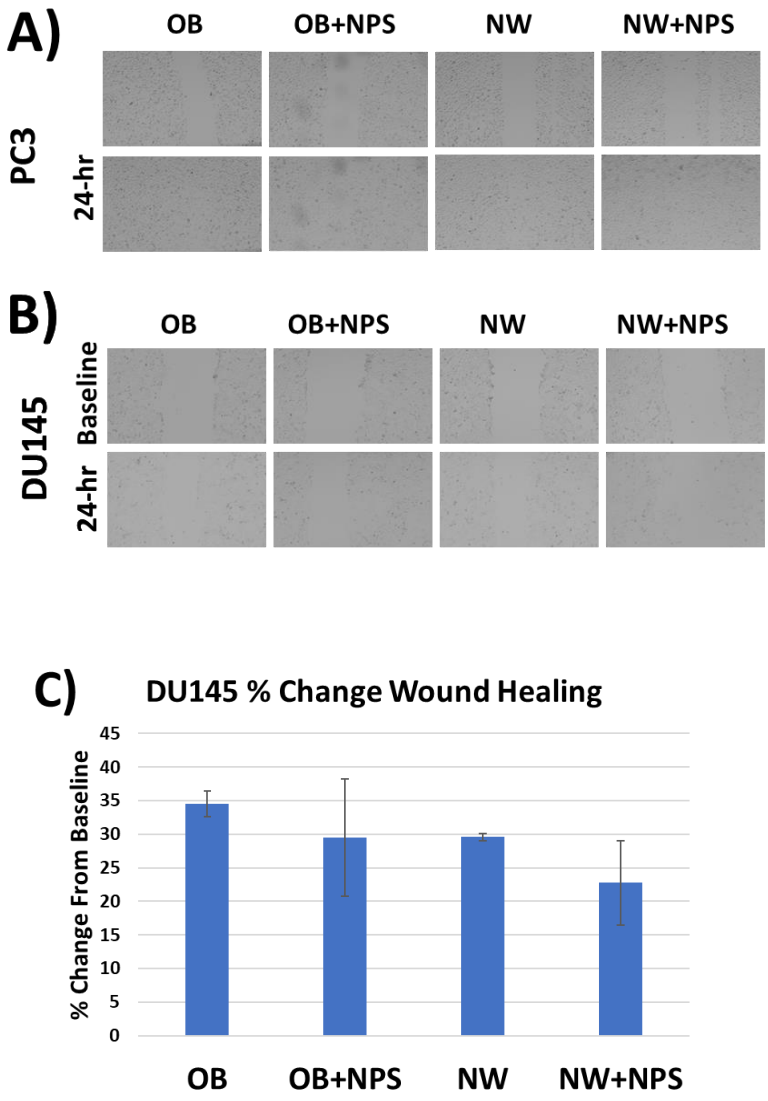


Figure 3: Wound Healing Assay. To determine differences in motility, a wound healing assay was performed. Cells were treated with OB (1%) or NW (1%) with or without 5µM NPS-2143 for 24 hours. Images of A) PC3 and B) DU145 cells were taken using the Cytation5 imaging reader. Graphic representation of C) DU145 cells is presented as percent change from baseline.

Effect of Obese Sera and CaSR on MMP-9 Activity

MMP-9 activity is important to breakdown the extracellular matrix, a process important for both evasion from the primary site and invasion into a secondary site. Zymography was used to determine MMP-9 proteolytic activity of PCa cells exposed to all treatment conditions. DU145 cells exposed to NW sera had decreased MMP-9 activity when treated with NPS-2143 compared to DU145 cells treated with NW sera alone. OB sera did not contribute to MMP-9 activity, with or without CaSR inhibition. This indicates CaSR does not contribute to obesity-mediated MMP-9 activation in PC3 or DU145 cells.

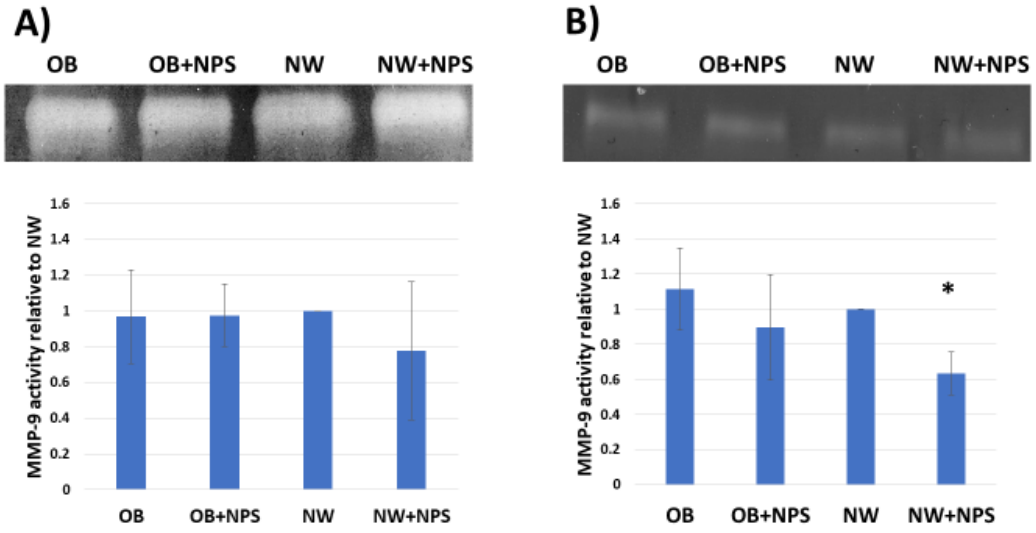


Figure 4: MMP-9 Activity. A) PC3 and B) DU145 cells were treated with obese (OB) sera, OB with NPS-2143 (OB+NPS), normal weight sera (NW), or NW with NPS-2143 (NW+NPS) for 1 hour and replaced with serum-free media. After 24 hours, conditioned media was harvested and concentrated 14-fold. Concentrated media was loaded on a gelatin SDS-PAGE. Proteolytic activity identified as clear bands and measured using densitometry. * $p < 0.05$ compared to NW.

Obese Sera Increases Invasive Capacity

BD BioCoat Matrigel Chambers were used to measure the invasive potential of PCa cells. The invasive capacity of PC3 cells was 43% greater ($p < 0.05$) in cells treated with OB sera compared to NW (figure 5). The invasive capacity of DU145 cells treated with OB sera was 90% greater ($p < 0.05$) than cells treated with NW sera (figure 5). DU145 and PC3 cells treated with OB sera and the CaSR inhibitor had similar invasive capacity to cells treated with NW sera alone. These results indicate obese sera increases the invasive phenotype, and CaSR may contribute to the obesity-mediated increase of the invasive phenotype of PC3 or DU145 cells.

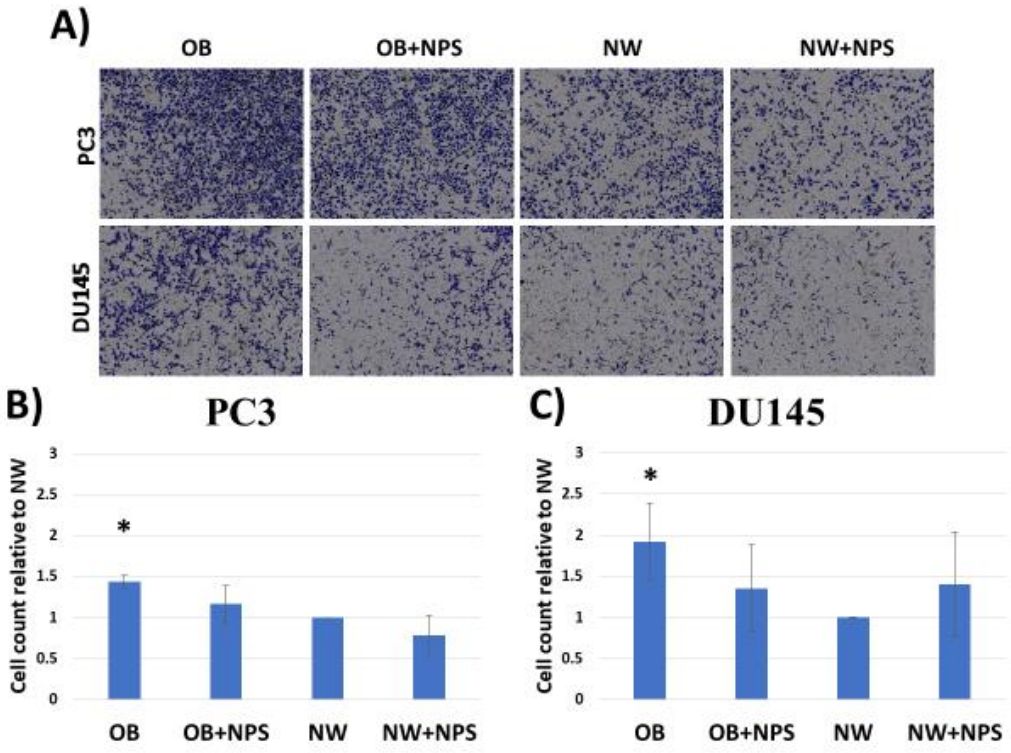


Figure 5. Obese Sera Increases Invasive Capacity of PC3 and DU145 Cells. Cells were treated in BD BioCoat Matrigel Chambers with obese sera (OB), OB with NPS-2143 (OB+NPS), normal weight sera (NW), NW with NPS-2143 (NW+NPS). Culture media was added to the bottom of the chamber as a chemoattractant. After 24 hours, cells were fixed, stained, and counted. A) Representative images of cells that migrated through the membrane were captured using Cytation 5. Cell count relative to NW are shown for B) PC3 and C) DU145 cells. * $p < 0.05$ compared to NW.

Effect of CaSR and Obese Sera on EMT

Epithelial-mesenchymal transition is important for PCa tumors to evade the primary tumor site, increasing risk for the development of metastasis. Two proteins that are important for the epithelial phenotype, promoting the formation of tight cell junctions are β-catenin and e-cadherin. De-localization of β-catenin and e-cadherin from the cell junctions indicates the mesenchymal phenotype. Immunofluorescence was used to

visualize differences in localization of β-catenin and e-cadherin. Therefore, these results are qualitative in nature, as quantitative differences were not measured. PC3 cells displayed minimal β-catenin localization at the cell membrane for all treatment groups (figure 6) demonstrating a mesenchymal phenotype. This could in part explain their highly metastatic phenotype as their cell junctions are low in an important protein for cell-to-cell contact. DU145 cells displayed dispersed β-catenin in cells treated with obese sera (figure 6). CaSR inhibition appeared to increase the localization of β-catenin to the cell junctions in DU145 cells, indicating CaSR may play a role of obesity-mediated β-catenin dispersion. DU145 cells treated with NW sera appeared to have increased levels of β-catenin at the cell junction in comparison to those treated with OB sera. DU145 cells treated with NW sera and the CaSR inhibitor showed lower levels of cytosolic protein in comparison to cells treated without NPS-2143. DU145 cells treated with OB sera appeared to have decreased localization of e-cadherin at the cell junctions in comparison to those treated with NW sera, which was attenuated with CaSR inhibition. These images indicate CaSR may contribute to the obesity-mediated mesenchymal phenotype of DU145 cells. DU145 cells treated with sera from normal weight males indicated increased protein expression at the cell junctions with low levels of e-cadherin in the cytosol. These results indicate obesity and CaSR may contribute to EMT in PC3 and DU145 cells. Further experiments are warranted to quantify differences in markers of EMT.

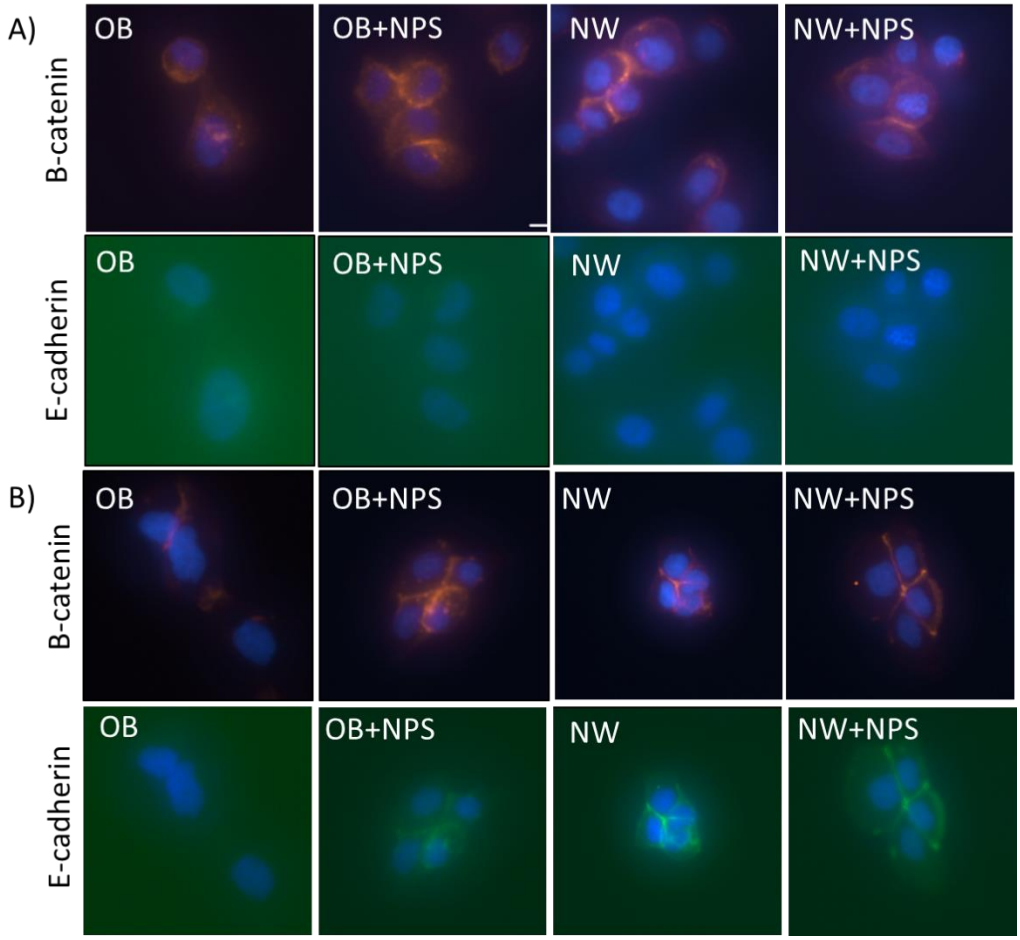


Figure 6. Visualization of EMT Proteins. A) PC3 and B) DU145 cells were exposed to obese sera (OB), OB with NPS-2143 (OB+NPS), normal weight sera (NW), or NW with NPS-2143 (NW+NPS) for 16-hours. Immunofluorescence was used to visualize localization of e-cadherin and beta catenin. PC3 and DU145 cells were treated with 5% obese sera, 5% normal weight sera with or without the addition of 5µM NPS-2143.

Gene Expression in PCa Cells

RT-PCR was used to measure differences in mRNA expression of several genes important to CaSR and PCa metastasis. PTHrP mRNA was reduced in PC3 cells treated with OB sera and NPS-2143 by 33.9% ($p < 0.05$) in comparison to NW indicating CaSR

may contribute to obesity-mediated PTHrP mRNA expression. TRPM7 mRNA expression was reduced in PC3 cells treated with OB sera by 35.8% ($p < 0.05$) and cells treated with OB sera and NPS-2143 by 54.86% ($p < 0.05$) in comparison to PC3 cells treated with NW sera, indicating obese sera may be protective against TRPM7 mRNA expression. Statistically significant differences were not observed in mRNA expression of CaSR, TRPC6, RANK or RANKL between all treatment conditions in PC3 cells. There were not statistically significant differences in mRNA expression of CaSR, PTHrP, TRPM7, TRPC6, RNAK, or RANKL in DU145 cells.

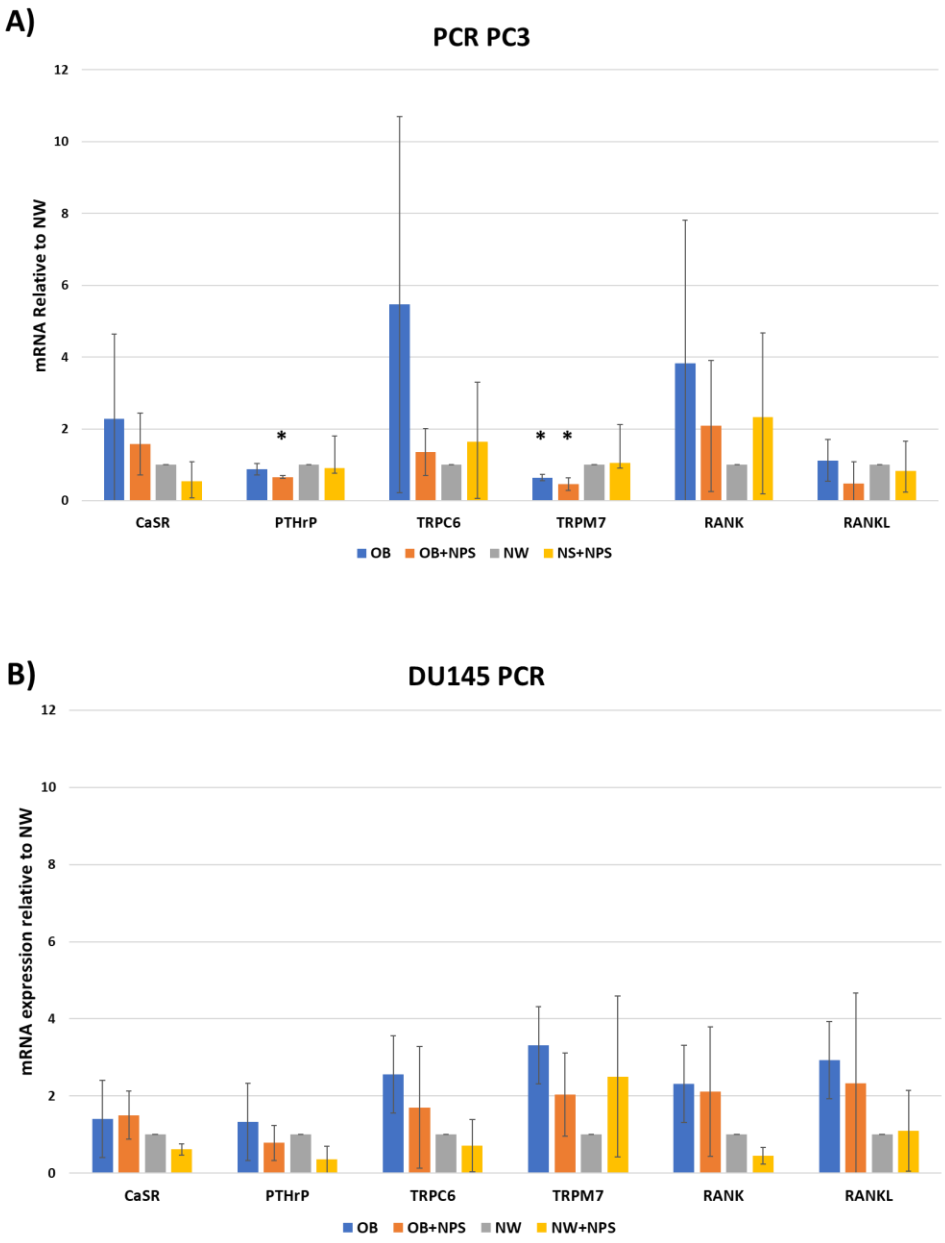
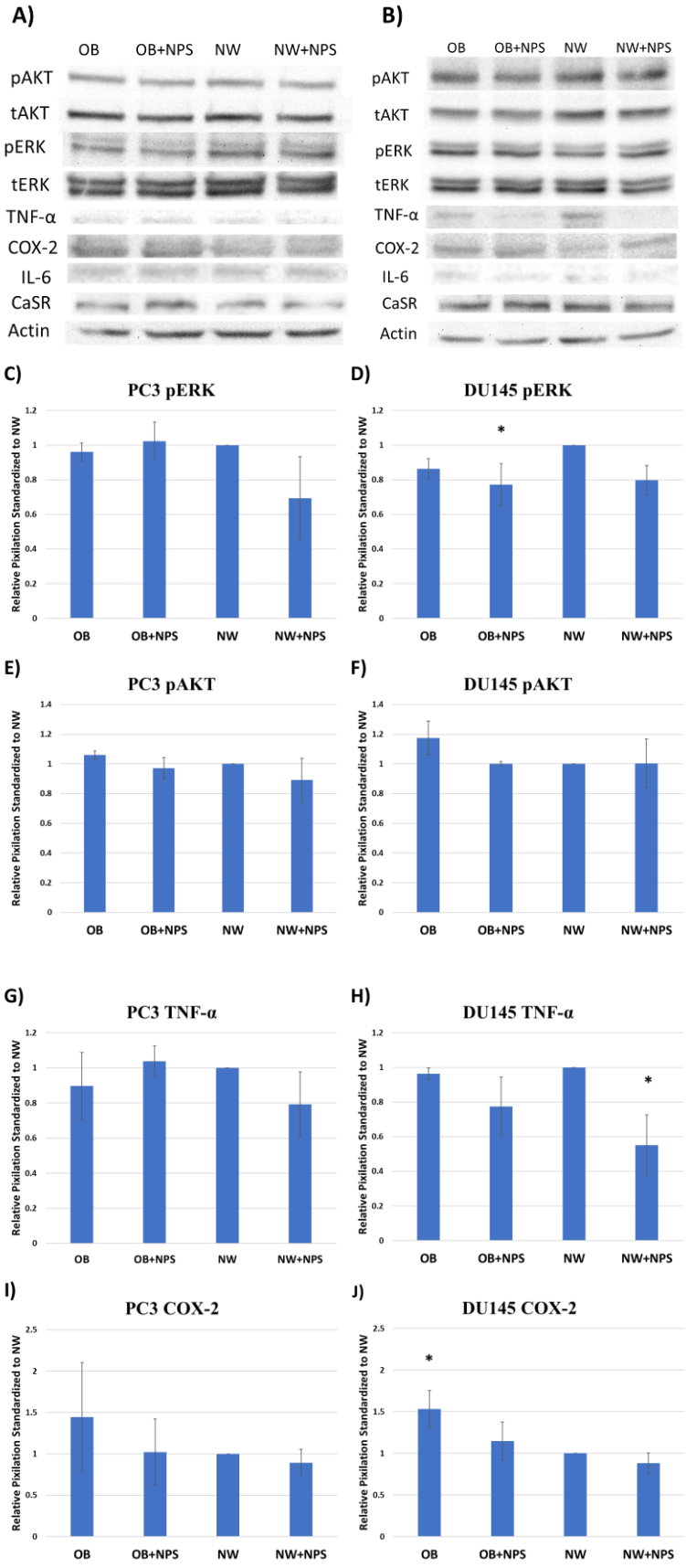


Figure 7: mRNA Expression. qRT-PCR was used to determine effects of CaSR on gene expression. A) PC3 and B) DU145 cells were treated with obese sera (OB), OB with NPS-2143 (OB+NPS), normal weight sera (NW), or NW+NPS) for one hour. * $p < 0.05$ compared to NW.

Inflammation and Tumorigenesis

Both obesity and CaSR have been associated with contributions to the inflammatory environment of PCa. Circulating obesity-mediated pro-inflammatory cytokines promote PCa tumorigenesis. To measure changes in inflammatory mediators and proteins important to tumorigenesis, western blot analysis was used. There were no statistically significant differences of pERK, pAKT, TNF-α, COX-2, IL-6, or CaSR in PC3 cells treated with OB sera or NPS-2143. OB sera increased protein expression of CaSR in DU145 cells treated with OB sera by 45.9% ($p < 0.05$) or OB sera with NPS-2143 by 45.0% ($p < 0.05$). This indicates OB sera may increase CaSR expression in androgen independent PCa cells that have not yet metastasized to the bone.

CaSR inhibition decreased phosphorylation of ERK by 22.8% ($p < 0.05$) in DU145 cells treated with OB sera in comparison to NW. TNF-α was reduced in DU145 cells by 44.9% ($p < 0.05$) in cells treated with NW sera and NPS-2143 in comparison to cells treated with NW sera alone. COX-2 and IL-6 expression was increased in DU145 cells by 53.3% ($p < 0.05$) and 64.6% ($p < 0.05$) respectively when treated with OB sera in comparison to NW.


A)
OB
OB+NPS
NW
NW+NPS
pAKT
tAKT
pERK
tERK
TNF-α
COX-2
IL-6
CaSR
Actin
B)
OB
OB+NPS
NW
NW+NPS
pAKT
tAKT
pERK
tERK
TNF-α
COX-2
IL-6
CaSR
Actin
C)
PC3 pERK
Relative Pixilation Standardized to NW
OB
OB+NPS
NW
NW+NPS
D)
DU145 pERK
*
Relative Pixilation Standardized to NW
OB
OB+NPS
NW
NW+NPS
E)
PC3 pAKT
Relative Pixilation Standardized to NW
OB
OB+NPS
NW
NW+NPS
F)
DU145 pAKT
Relative Pixilation Standardized to NW
OB
OB+NPS
NW
NW+NPS
G)
PC3 TNF-α
Relative Pixilation Standardized to NW
OB
OB+NPS
NW
NW+NPS
H)
DU145 TNF-α
*
Relative Pixilation Standardized to NW
OB
OB+NPS
NW
NW+NPS
I)
PC3 COX-2
Relative Pixilation Standardized to NW
OB
OB+NPS
NW
NW+NPS
J)
DU145 COX-2
*
Relative Pixilation Standardized to NW
OB
OB+NPS
NW
NW+NPS

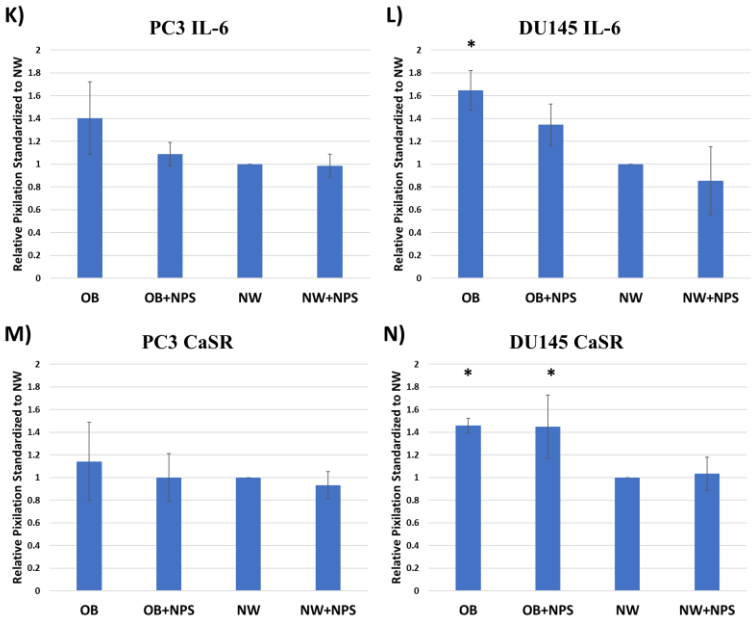


Figure 8: Effects of Sera and CaSR on Protein Expression in PCa Cells. A) PC3 and B) DU145 cells were treated with obese sera (OB), OB with NPS-2143 (OB+NPS), normal weight sera (NW), or NW with NPS-2143 (NW+NPS). Phosphorylated ERK (pERK) was measured in C) PC3 and D) DU145 cells following exposure to treatments for 15 minutes. Phosphorylated AKT (pAKT) was measured in E) PC3 and F) DU145 cells following treatment for 15 minutes. G) PC3 and H) DU145 cells were treated for 4 hours and assessed for TNF-α expression. I) PC3 and J) DU145 cells were treated for 4 hours, then COX-2 expression was determined. K) PC3 and L) DU145 cells were treated for 4 hours and assessed for IL-6. M) PC3 and N) DU145 cells were treated for 4 hours and CaSR expression was determined. Data is reported as a ratio of protein of interest to actin and pixilation relative to NW. * $p < 0.05$ compared to NW.

Discussion

The American Institute of Cancer Research has linked 13 cancers and advanced PCa to obesity.[11] It is important to elucidate mechanisms that increase the risk for obesity-mediated advanced PCa to establish better prevention strategies and treatment plans. Mechanisms that have been linked to obesity and tumor promoting inflammation include the dysregulation of cell signaling, alterations to the microenvironment, systemic inflammation, and disruption of the vasculature.[15] CaSR was identified as a potential

target for treatment, or a biomarker that could alter the treatment plan for those diagnosed with PCa. CaSR was recognized as a potential target for obesity-mediated PCa progression due to its previously reported response to obesity-mediated inflammatory mediators.[28,54,55] CaSR is associated with lethal PCa tumors and tumors that have metastasized to the bone, indicating a positive CaSR biopsy may warrant a radical prostatectomy.[69,70]

In this study, several intermediates in which CaSR may contribute to obesity-mediated PCa progression have been identified. Treatment with OB sera caused the upregulation of CaSR expression in DU145 cells which an interesting result because they were derived from a brain metastasis, while PC3 cells were derived from a bone metastasis. This may suggest cells that have established a tumor in the bone have already altered CaSR expression or CaSR-mediated cell signaling. It is important to elucidate downstream effects of CaSR as they have been shown to contribute to inflammation and increase bone remodeling, which increases risk for bone metastasis. DU145 cells increased expression of the inflammatory mediators COX-2 and IL-6 following treatment with OB sera without CaSR inhibition. DU145 cells treated with OB sera and the CaSR inhibitor had similar expression of CaSR to cells treated with NW sera, indicating CaSR may play an important role in mediating inflammation in androgen independent PCa cells that have not yet metastasized to the bone. It has been previously reported that CaSR expression is increased in response to inflammation in other cell types, and is at least partially mediated through NF-κB translocation to the nucleus which is important for obesity-mediated aggressive PCa phenotype.[28,55] Increased NF-κB activity in response to

high levels of TNF-α in PCa cells leads to increased VEGF, IL-6, and COX2, which are important factors for obesity-mediated PCa progression.[19]

This study identified decreased PTHrP mRNA expression in PC3 cells treated with OB sera and NPS-2143. PTHrP plays an important role in bone remodeling and PCa metastasis to the bone, and could help identify future therapeutic targets including PTHrP itself or factors promoting PTHrP excretion or molecules that are affected by PTHrP. TPRM7 had decreased mRNA expression when treated with OB sera with or without NPS-2143. This indicates obesity may be protective of TRPM7 overexpression which has been associated with both proliferation, and invasion and migration. TRPM7 is important to maintaining calcium and magnesium homeostasis in PCa cells, and increased calcium to magnesium ratio increases calcium influx and proliferation.[59,73] Obesity also contributes to the dysregulation of mineral homeostasis in the cytoplasm and organelles indicating a potential link between obesity and PCa tumorigenesis.[65] Cholesterol also increases calcium influx through TRPM7 in PCa cells, while activating AKT and ERK, and decreasing e-cadherin expression.[60] Although TRPM7 mRNA was not increased in this study, OB sera may be increasing TRPM7 activation which needs to be measured with electrophysiological assays. mRNA levels of CaSR, TRPC6, RANK, and RANKL were not statistically different in any treatment groups. However, CaSR protein levels were increased in DU145 cells treated with OB sera, indicating OB sera may increase translation of mRNA into protein. TRPC6 activation is associated with proliferation of PCa cells through PKC activation and NFAT translocation to the nucleus.[56] The expression of TRPC6 may be independent of obesity and CaSR, however its activity may

be increased with obesity-derived inflammatory factors or CaSR activity, which would need to be measured using electrophysiological assays.

Obese sera led to a visualization of β-catenin and e-cadherin de-localized from cell-to-cell junctions and dispersed throughout the cytosol. This indicates transition from the epithelial to mesenchymal transition, thus increasing risk for PCa cells to enter into circulation and form a secondary tumor. These results are consistent with previous studies where PCa cells were exposed to sera from obese mice resulting in de-localization of β-catenin and e-cadherin from the cell membrane.[72] CaSR inhibition appeared to attenuate this response, indicating a potential contribution to EMT. Future studies should be designed to measure translocation and expression of β-catenin and e-cadherin, because this study did not quantify differences. It is also important to measure differences in other markers important for the mesenchymal phenotype including vimentin and n-cadherin. Obese sera increased the invasive capacity of both PC3 and DU145 cells. Cells treated with OB sera and CaSR inhibition had similar invasive capacity of cells treated with NW sera, indicating CaSR may contribute to the obesity-mediated invasive phenotype of PCa cells. PCa cells including DU145, LNCaP, and PacMetUT1 exposed to sera from obese mice have previously been shown to increase invasive capacity.[72] MMP-9 activity was only different in DU145 cells treated with NW sera and NPS-2143. This indicates CaSR likely does not contribute to obesity-mediated MMP-9 activity in this model. Our study used 24-hour conditioned media, and PC3 cells had high levels of MMP-9 activity with no differences between groups. However, future studies with different time points and interfering RNA (RNAi) could show attenuation with CaSR knockdown. The use of

individual cytokines instead of sera will also help to identify the link between CaSR and inflammation-mediated PCa progression.

Diets incorporating deficient or excessive amounts of calcium have been associated with increased risk for aggressive PCa or PCa recurrence following radical prostatectomy.[6] Bernichstein et al.[62] established in a mouse model where high calcium diets increased both CaSR and TRPC6. There are proposed mechanisms of diets high in calcium and PCa risk such as increased free IGF-1, associated with increased consumption of dairy products,[5] and decreased formation of active vitamin D 1,25-dihydroxy vitamin D.[3,4] Other studies have identified a link between obesity-derived inflammatory factors (IL-1β, IL-6, and TNF-α) and increased CaSR expression in various tissues including the parathyroid, thyroid, kidney, and adipocytes.[28,55]

CaSR has previously been associated with poor outcomes in various cancers such as PCa, breast cancer, and Leydig cancer.[10] The traditional mechanism for PCa and breast cancer includes increased PTHrP leading to increased bone turnover.[10] Evidence of increased CaSR in response to obesity-mediated inflammatory mediators (IL-1β, IL-6, and TNF-α) in various tissues, and increased CaSR in PCa tumors that are derived from the bone and lethal tumors indicates a potential target to break the link between obesity and advanced PCa.

The model used for this study utilized pooled human sera, which has many factors that may stimulate but also inhibit the inflammatory response. The CaSR inhibitor (NPS-2143) used has limitations such as potential off-target effects and unintended modulation of the CaSR protein. It is important to target specific obesity-mediated inflammatory

factors found in sera to further understand the contribution of CaSR to obesity-mediated PCa progression. Using RNAi to knockdown CaSR will also be a valuable tool to assess the impact of specific inflammatory factors including IGF-1, IL-6, and TNF-α.

In summary, this research has identified several pro-inflammatory intermediates and physiological phenotypes in which CaSR may contribute to obesity-mediated PCa progression. COX-2 and IL-6 expression increased when exposed to OB sera, however when exposed to OB sera and NPS-2143 expression was similar to that of NW sera alone. OB sera appeared to de-localize β-catenin from the cell membranes of PC3 and DU145 cells and de-localize e-cadherin from DU145 cells. OB sera also increased the invasive capacity of both PC3 and DU145 cells, however exposure to OB sera and NPS-2143 caused similar invasive capacity as exposure to NW sera alone. CaSR may be a critical component to the development of advanced PCa and obesity-mediated PCa progression. Its interaction with obesity-associated inflammatory factors may provide viable targets for decreasing risk for obesity-mediated advanced PCa.

CHAPTER III

FUTURE DIRECTIONS

For physiological outcomes, differences in cell viability were not observed as expected. It would be valuable to perform a growth curve to determine differences in cell growth as opposed to cell viability. A longer timepoint of 96 hours instead of 72 hours may have also achieved differences that were not seen. Differences in invasive capacity were observed using Matrigel Invasion Chambers. It could be beneficial to expand this research into various chemokines and adhesion molecules that are important for cell migration. CaSR modulates migration and invasion of medullary thyroid carcinoma cells through the formation of a functional protein complex with β1-containing integrins which bind to extracellular matrix binding proteins.[74] CaSR upregulates e-cadherin in colon cancer cells which is protective of the epithelial to mesenchymal transition and thus migration.[24] However the effects of CaSR on e-cadherin may be the opposite in PCa cells, as CaSR expression has been hypothesized to be protective in colon cancer, but promoting metastatic cancer in PCa.[68] Identifying other chemokines and adhesion molecules that may complex with CaSR could provide targets for interventions. Preliminary data could be obtained using wound healing assay. Those results could warrant the use of invasion chambers to measure migratory capacity. Expanding this to co-culture with osteocytes would also be valuable to determine effects increasing risk for bone metastasis. PCa cells expressing CaSR secrete PTHrP which is known to attract PCa cells to osteocytes. It would be important to ascertain if obese sera, or inflammatory

factors are upregulating the excretion or production of PTHrP, which would demonstrate the importance of obesity in the progression of metastatic PCa.

Obesity causes hypoxia in white adipose tissue and is associated with metastatic cancer and poor prognosis.[75] The hypoxic environment, leptin, NF-κB, and IGF-1 stimulate HIF-1 which promotes tumorigenesis.[16,75] The result of hypoxia along with increased VEGF is increased vasculature which is required to deliver oxygen and nutrients to the tumor and increase options for the transport mesenchymal stromal cells.[75] Hypoxic adipose tissue also releases inflammatory mediators such as TNF-α.[16] The obesity-induced hypoxic environment also causes an acidic environment in which tumor cells are better able to adapt than normal cells, giving them the advantage for metabolic and survival factors.[76,77] Changes in pH are also caused by bone remodeling and can activate CaSR.[10,78] The endosteal surface in which tumor cells invade for bone metastasis have higher level of calcium which has been shown to attract CaSR positive hematopoietic stem cells.[24] Previous research in our lab has identifies CaSR appears to be upregulated when nutrients are depleted (data not shown). It may be that PCa cells use CaSR as a survival protein in response to reduced nutrient supply, recognizing if they more calcium is not found, they will not survive. Cells in the center of a tumor are exposed to lower levels of oxygen, nutrients, pH.[79,80] These alterations may upregulate CaSR to levels that increase inflammation, motility, and PTHrP excretion. Experiments could be set designed to assess differences in CaSR and PTHrP excretion when pH, oxygen, and nutrient levels are altered, thus simulating obesity-induced changes to the microenvironment. Differences could be measured in other survival proteins, transcription factors and migration-associated chemokines.

Co-culture with osteocytes would help to learn more about how obesity-derived inflammatory factors and CaSR may increase bone turnover, thus increasing risk for bone metastasis. Research from Herroon et al.,[81] indicates FABP4 and IL-1β are increased in PCa cells exposed to bone barrow adipocytes. Pre-adipocytes and adipocytes express CaSR and are responsive to obesity-mediated pro-inflammatory cytokines including TNF-α and IL-1β.[82] In adipocytes, CaSR activation has been associated with activation of ERK, NF-κB, and PI3K which increased both adipogenesis and tumorigenesis.[82] Determining if diet-induced obesity increases CaSR in both PCa cells and bone marrow adipocytes leading to increased invasion and metastasis could help to further understand the correlation between obesity and invasive PCa. Obtaining adipocytes and bone marrow stromal cells from humans and stratifying by BMI could give insight through PCa/adipocyte/osteocyte co-culture models as to why lethal PCa is more likely in obese individuals.

One important aspect of the progression in PCa which correlates with ion channels is the decrease of Mg2+ and increase of calcium.[59] TRPM7 channels were shown to be upregulated in PCa cell in comparison to control cells as well as age matched PCa patients.[59] TRPM7 mRNA was increased in DU145 cells treated with obese sera, and reduced with CaSR inhibition, however differences were not statistically significant. Exposing cells to various levels of Mg2+ and calcium could identify further pathways where dietary intervention could help to prevent the progression of PCa. High Ca2+ to Mg2+ ratios have been shown to increase calcium influx and proliferation of PCa cells. If increased levels of Mg2+ could mitigate some of the metastatic effects of CaSR by inhibiting negative effects of CaSR activation, then dietary changes including increased

Mg2+ intake and Ca2+ intake that does not exceed dietary recommendations. It has been previously shown that Mg2+ binds to CaSR, but the downstream effects in PCa cells is not known.[83] Identifying physiological and cell signaling changes in PCa cells upon CaSR activation and ion channel activation with various Ca2+: Mg2+ ratios could identify potential interventions that might inhibit PCa tumorigenesis.

In medullary thyroid carcinoma cells, CaSR has been shown to bind to members of integrins of extracellular binding proteins.[74] The coupling of CaSR to integrin proteins led to increased migration and cellular adhesion.[74] IGFBPs also exhibit effects on integrins in an IGF-1 independent manner.[44] Obesity does modulate IGFBPs, some of which are upregulated and some of which are downregulated. It would be valuable to identify if obesity or obesity-mediated inflammatory factors upregulate and promote the functional coupling of CaSR to molecules important for adhesion and metastasis.

Differences in EMT proteins were visualized. It would be important elucidate further EMT protein alterations with CaSR and inflammatory factors, and in co-culture models with adipocytes and osteocytes. Other measures besides visualization are important, such as quantifying alterations in mRNA and protein levels of important EMT markers such as decreased E-cadherin, increased N-cadherin, Snail, or vimentin. Snail is a transcription factor that permits the expression of genes required for the mesenchymal phenotype.[84]

In summary, this study reviewed a broad spectrum of potential alterations to PCa cells associated with obesity and obesity-induced inflammatory mediators. It is important to continue study in this field to identify targets for therapeutic remedies. Because CaSR

is overexpressed in metastatic PCa, biopsies exposing CaSR overexpression may warrant radical prostatectomy. CaSR inhibition through traditional pharmaceuticals is not warranted due to the ubiquitous expression of CaSR. However, localized treatment through nano-delivery systems may be developed to ensure CaSR inhibition is localized to the prostate. Downstream signaling of CaSR may be further studied to develop targets that could block the negative effects of CaSR activation. Activators of CaSR may also be modified through dietary modifications or reduction of BMI, which improve calcium homeostasis in the tumor microenvironment resulting in decreased CaSR activation in PCa cells. CaSR is upregulated in several organs by obesity-mediated inflammatory cytokines, and is associated with metastatic and lethal tumors. Thus, developing therapies targeting CaSR in obese individuals could reduce risk for metastasis and lethal PCa.

LITERATURE CITED

1. Miller KD, Siegel RL, Lin CC, et al. Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin*. 2016;66(4):271-289. doi:10.3322/caac.21349.

2. World Cancer Research Fund. Diet , nutrition , physical activity and prostate cancer. 2014:50. http://www.wcrf.org/sites/default/files/Prostate-Cancer-2014-Report.pdf.

3. Rodriguez C, McCullough ML, Mondul AM, et al. Calcium, dairy products, and risk of prostate cancer in a prospective cohort of United States men. *Cancer Epidemiol Biomarkers Prev*. 2003;12(7):597-603. http://ovidsp.ovid.com/ovidweb.cgi?T=JS&CSC=Y&NEWS=N&PAGE=fulltext&D=med4&AN=12869397%5Cnhttp://resolver.ebscohost.com/openurl?issn=10559965&Volume=12&issue=7&spage=597&title=Calcium,+dairy+products,+and+risk+of+prostate+cancer+in+a+prospective+cohort+of.

4. Lokeshwar BL, Schwartz GG, Selzer MG, et al. Inhibition of prostate cancer metastasis in vivo: a comparison of 1,23-dihydroxyvitamin D (calcitriol) and EB1089. *Cancer Epidemiol Biomarkers Prev*. 1999;8(3):241-248. http://www.ncbi.nlm.nih.gov/pubmed/10090302.

5. Giovannucci E, Pollak M, Liu Y, et al. Nutritional predictors of insulin-like growth factor 1 and their relationships to cancer in men. *Cancer Epidemiol Biomarkers Prev*. 2003;12(February):84-89.

6. Binder M, Shui I, Wilson K, et al., Calcium intake, polymorphisms of the calcium-sensing receptor, and recurrent/aggressive protate cancer. *Cancer Causes Control*. 2015;26(12):1751-1759.

7. Rybak AP, Bristow RG, Kapoor A. Prostate cancer stem cells: deciphering the origins and pathways involved in prostate tumorigenesis and aggression. *Oncotarget*. 2015;6(4):1900-1919. doi:10.18632/oncotarget.2953.

8. American Cancer Society. Prostate cancer what is prostate cancer. Atlanta, Ga: American Cancer Society; 2016;1-88.

9. American Cancer Society. Cancer facts and figures 2016. Atlanta Ga: American Cancer Society; 2016;1-72. doi:10.1097/01.NNR.0000289503.22414.79.

10. Kingsley LA, Fournier PGJ, Chirgwin JM, Guise TA. Molecular biology of bone metastasis. *Mol Cancer Ther*. 2007;6(10):2609-2617. doi:10.1158/1535-7163.MCT-07-0234.

11. Secretan BL, Scoccianti C, Loomis D, et al., Body fatness and cancer - viewpoint of the IARC working group. *N Engl J Med.* 2016;375(8):794-798.

12. Cao Y, Ma J. Body mass index, prostate cancer-specific mortality, and biochemical recurrence: A systematic review and meta-analysis. *Cancer Prev Res*. 2011;4(4):486-501. doi:10.1158/1940-6207.CAPR-10-0229.

13. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell*. 2011;144(5):646-674. doi:10.1016/j.cell.2011.02.013.

14. Donohoe CL, Lysaght J, O'Sullivan J, Reynolds J V. Emerging concepts linking obesity with the hallmarks of cancer. *Trends Endocrinol Metab*. 2016;xx:1-17. doi:10.1016/j.tem.2016.08.004.

15. Trulson MF, Clancy RE, Stare FJ. Advances in nutrition and dietetics. *Practitioner*. 1962;189:505-512. doi:10.1007/978-3-642-38007-5.

16. Pérez-Hernández AI, Catalán V, Gómez-Ambrosi J, Rodríguez A, Frühbeck G. Mechanisms linking excess adiposity and carcinogenesis promotion. *Front Endocrinol (Lausanne)*. 2014;5(MAY):1-17. doi:10.3389/fendo.2014.00065.

17. Ricardo JT, Ribeiro R, Catia PD, el al., Tumor cell-educated periprostatic adipose tissue acquires an aggressive cancer-promoting secretory profile. *Cell Physiol Biochem.* 2012;29:233-240.

18. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature*. 2006;441(5):431-436. doi:10.1038/nature04870.

19. Garg R, Blando J, Perez CJ, Wang H, Benavides FJ, Kazanietz MG. Activation of nuclear factor κB (NF-κB) in prostate cancer is mediated by protein kinase c ε (PKCε). *J Biol Chem*. 2012;287(44):37570-37582. doi:10.1074/jbc.M112.398925.

20. Taylor B, Schultz N, Hieronymus H. Integrative genomic profiling of human prostate cancer. *Cancer Cell*. 2010;18(1):11-22. doi:10.1016/j.ccr.2010.05.026.Integrative.

21. Bluher M, Mantzoros CS. From leptin to other adipokines in health and disease: Facts and expectations at the beginning of the 21st century. *Metabolism*. 2015;64(1):131-145. doi:10.1016/j.metabol.2014.10.016.

22. Alshaker H, Sacco K, Alfraidi A, Muhammad A, Winkler M, Pchejetski D. Leptin signalling, obesity and prostate cancer: molecular and clinical perspective on the old dilemma. *Oncotarget*. 2015;6(34):35556-35563. doi:10.18632/oncotarget.5574.

23. Cao Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest*. 2007;117(9):2362-2368. doi:10.1172/JCI32239.

24. Sarkar P, Kumar S. Calcium sensing receptor modulation for cancer therapy. *Asian Pacific J Cancer Prev*. 2012;13(8):3561-3568. doi:10.7314/APJCP.2012.13.8.3561.

25. Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Brown EM, Jennifer L. Ca2+ -sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells. 2001:1267-1274.

26. Chang W, et al., Modulator of Skeletal Development. 2013;1(35):1-23. doi:10.1126/scisignal.1159945.The.

27. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol*. 2009;1(6):1-10. doi:10.1101/cshperspect.a001651.

28. Canaff L, Hendy GN. Calcium-sensing receptor gene transcription is up-regulated by the proinflammatory cytokine, interleukin-1β: Role of the NF-κB pathway and κB elements. *J Biol Chem*. 2005;280(14):14177-14188. doi:10.1074/jbc.M408587200.

29. Zhang Y, Lapidus RG, Liu P, et al. Small molecule therapeutics targeting IkB kinase b/NF-kB signaling in human prostate cancer by a novel IkB kinase b inhibitor CmpdA. *Mol Cancer Ther*. 2016;15(7):1504-1514. doi:10.1158/1535-7163.MCT-15-0999.

30. Mathes E, O'Dea EL, Hoffmann A, Ghosh G. NF-κB dictates the degradation pathway of IκBα. *EMBO J*. 2008;27(9):1357-1367. doi:10.1038/emboj.2008.73.

31. McCubrey JA, Steelman LS, Chappell WH, et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta - Mol Cell Res*. 2007;1773(8):1263-1284. doi:10.1016/j.bbamcr.2006.10.001.

32. Kumar B, Koul S, Khandrika L, Meacham RB, Koul HK. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. *Cancer Res*. 2008;68(6):1777-1785. doi:10.1158/0008-5472.CAN-07-5259.

33. McMurray F, Patten DA, Harper ME. Reactive oxygen species and oxidative stress in obesity recent findings and empirical approaches. *Obesity*. 2016;24(11):2301-2310. doi:10.1002/oby.21654.

34. Nguyen DP, Li J, Tewari AK. Inflammation and prostate cancer: The role of interleukin 6 (IL-6). *BJU Int*. 2014;113(6):986-992. doi:10.1111/bju.12452.

35. Bellido T, Borba VZC, Roberson P, Manolagas SC. Activation of the janus kinase / STAT (signal transducer and activator of transcription) signal transduction pathway by interleukin-6-type cytokines promotes osteoblast differentiation. *Society*. 1997;138(9):3666-3676. doi:10.1210/en.138.9.3666.

36. Shukla S, MacLennan GT, Hartman DJ, Fu P, Resnick MI, Gupta S. Activation of PI3K-Akt signaling pathway promotes prostate cancer cell invasion. *Int J Cancer*. 2007;121(7):1424-1432. doi:10.1002/ijc.22862.

37. Steiner H, Berger AP, Godoy-Tundidor S, et al. An autocrine loop for vascular endothelial growth factor is established in prostate cancer cells generated after prolonged treatment with interleukin 6. *Eur J Cancer*. 2004;40(7):1066-1072. doi:10.1016/j.ejca.2003.11.033.

38. Giannoni E, Bianchini F, Masieri L, et al. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. *Cancer Res*. 2010;70(17):6945-6956. doi:10.1158/0008-5472.CAN-10-0785.

39. Hobisch A, Eder IE, Putz T, et al. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. *Cancer Res*. 1998;58(20).

40. Lee SO, Lou W, Hou M, Miguel F De, Gerber L, Gao AC. Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. *Clin Cancer Res.* 2003;9(1):370-376.

41. Font-Burgada J, Sun B, Karin M. Obesity and Cancer: The oil that feeds the flame. *Cell Metab*. 2016;23(1):48-62. doi:10.1016/j.cmet.2015.12.015.

42. Fahrenholtz C, Greene A, Beltran P, Burnstein K. A novel calcium-dependent mechanism of acquired resistance to IGF-1 receptor inhibition in prostate cancer cells. *Oncotarget*. 2014;5(19):11-14. doi:2346.

43. Freedland SJ, Aronson WJ. Examining the relationship between obesity and prostate cancer. *Rev Urol*. 2004;6(2):73-81. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1550782&tool=pmcentrez&rendertype=abstract.

44. Denduluri SK, Idowu O, Wang Z, et al. Insulin-like growth factor (IGF) signaling intumorigenesis and the development ofcancer drug resistance. *Genes Dis*. 2015;2(1):13-25. doi:10.1016/j.gendis.2014.10.004.

45. Yoneda T, Hiraga T. Crosstalk between cancer cells and bone microenvironment in bone metastasis. *Biochem Biophys Res Commun*. 2005;328(3):679-687. doi:10.1016/j.bbrc.2004.11.070.

46. Breuksch I, Weinert M, Brenner W. The role of extracellular calcium in bone metastasis. *J Bone Oncol*. 2016;5(3):143-145. doi:10.1016/j.jbo.2016.06.004.

47. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem*. 2000;69(1):145-182. doi:10.1146/annurev.biochem.69.1.145.

48. Bieniek J, Childress C, Swatski MD, Yang W. COX-2 inhibitors arrest prostate cancer cell cycle progression by down-regulation of kinetochore/centromere proteins. *Prostate*. 2014;74(10):999-1011. doi:10.1002/pros.22815.

49. Ji R, Chou C, Xu W, Chen X, Woodward DF, Regan JW. EP1 Prostanoid receptor coupling to Gi/o up-regulates the expression of hypoxia-inducible factor-1 through activation of a phosphoinositide-3 kinase signaling pathway. *Mol Pharmacol*. 2010;77(6):1025-1036. doi:10.1124/mol.110.063933.

50. Breyer MD, Breyer RM. G protein-coupled prostanoid receptors and the kidney. *Annu Rev Physiol.* 2001;63:579-605.

51. Kameda T, Mano H, Yamada Y, et al. Calcium-sensing receptor in mature osteoclasts, which are bone resorbing cells. *Biochem Biophys Res Commun*. 1998;245(2):419-422. doi:10.1006/bbrc.1998.8448.

52. Lines C, Sanders JL, Chattopadhyay N, et al. Extracellular calcium-sensing receptor expression and its potential role in regulating parathyroid hormone-related peptide secretion in human breast cancer. *Endo.* 2015;141(12):0-7.

53. Yano S, Macleod RJ, Chattopadhyay N, et al. Calcium-sensing receptor activation stimulates parathyroid hormone-related protein secretion in prostate cancer cells: role of epidermal growth factor receptor transactivation. *Bone*. 2004;35(3):664-672. doi:10.1016/j.bone.2004.04.014.

54. Canaff L, Zhou X, Hendy GN. The proinflammatory cytokine, interleukin-6, up-regulates calcium-sensing receptor gene transcription via Stat1/3 and Sp1/3. *J Biol Chem*. 2008;283(20):13586-13600. doi:10.1074/jbc.M708087200.

55. Cifuentes M, Fuentes C, Mattar P, et al. Obesity-associated proinflammatory cytokines increase calcium sensing receptor (CaSR) protein expression in primary human adipocytes and LS14 human adipose cell line. *Arch Biochem Biophys*. 2010;500(2):151-156. doi:10.1016/j.abb.2010.05.033.

56. Déliot N, Constantin B. Plasma membrane calcium channels in cancer: alterations and consequences for cell proliferation and migration. *Biochim Biophys Acta - Biomembr*. 2015;1848(10):2512-2522. doi:10.1016/j.bbamem.2015.06.009.

57. Capiod T, Rosado J. Calcium entry pathways in non-excitable cells: extracellular calcium has multiple targets to control cell proliferation to control cell proliferation. *Advances in Experimental and Medicine and Biology.* 2016;898:144-156. doi:10.1007/978-3-319-26974-0.

58. Dubois C, Vanden Abeele F, Lehen'kyi V, et al. Remodeling of channel-forming ORAI proteins determines an oncogenic switch in prostate cancer. *Cancer Cell*. 2014;26(1):19-32. doi:10.1016/j.ccr.2014.04.025.

59. Sun Y, Selvaraj S, Varma A, Derry S, Sahmoun AE, Singh BB. Increase in serum Ca2+/Mg2+ ratio promotes proliferation of prostate cancer cells by activating TRPM7 channels. *J Biol Chem*. 2013;288(1):255-263. doi:10.1074/jbc.M112.393918.

60. Sun Y, Sukumaran P, Varma A, Derry S, Sahmoun AE, Singh BB. Cholesterol-induced activation of TRPM7 regulates cell proliferation, migration, and viability of human prostate cells. *Biochim Biophys Acta - Mol Cell Res*. 2014;1843(9):1839-1850. doi:10.1016/j.bbamcr.2014.04.019.

61. Raphaël M, Lehen'kyi V, Vandenberghe M, et al. TRPV6 calcium channel translocates to the plasma membrane via ORAI1-mediated mechanism and controls cancer cell survival. *Proc Natl Acad Sci U S A*. 2014;111(37):E3870-9. doi:10.1073/pnas.1413409111.

62. Bernichtein S, Pigat N, Barry Delongchamps N, et al. Vitamin D3 prevents calcium-induced progression of early-stage prostate tumors by counteracting TRPC6 and calcium sensing receptor upregulation. *Cancer Res*. 2017;77(2):355-365. doi:10.1158/0008-5472.CAN-16-0687.

63. Monet M, Lehen'kyi V, Gackiere F, et al. Role of cationic channel TRPV2 in promoting prostate cancer migration and progression to androgen resistance. *Cancer Res*. 2010;70(3):1225-1235. doi:10.1158/0008-5472.CAN-09-2205.

64. Tyutyunnykova A, Telegeev G, Dubrovska A. The controversial role of phospholipase C epsilon (PLCε) in cancer development and progression. *J Cancer*. 2017;8(5):716-729. doi:10.7150/jca.17779.

65. Arruda AP, Hotamisligil GS. Calcium homeostasis and organelle function in the pathogenesis of obesity and diabetes. *Cell Metab*. 2015;22(3):381-397. doi:10.1016/j.cmet.2015.06.010.

66. Porter MP, Stanford JL. Obesity and the risk of prostate cancer. *Prostate*. 2005;62(April 2004):316-321. doi:10.1002/pros.20121.

67. Allott EH, Masko EM, Freedland SJ. Obesity and prostate cancer: weighing the evidence. *Eur Urol*. 2013;63(5):800-809. doi:10.1016/j.eururo.2012.11.013.

68. Chakravarti B, Dwivedi SKD, Mithal A, Chattopadhyay N. Calcium-sensing receptor in cancer: good cop or bad cop. *Endocrine*. 2009;35(3):271-284. doi:10.1007/s12020-008-9131-5.

69. Ahearn TU, Tchrakian N, Wilson KM, et al. Calcium-sensing receptor tumor expression and lethal prostate cancer progression. *J Clin Endocrinol Metab*. 2016;101(6):2520-2527. doi:10.1210/jc.2016-1082.

70. Feng J, Xu X, Li B, et al. Prostate cancer metastatic to bone has higher expression of the calcium-sensing receptor (CaSR) than primary prostate cancer. *Recept Clin Investig*. 2015;1(6):1-14. doi:10.14800/rci.270.

71. Miethe C, Nix H, Martin R, et al. Silibinin reduces the impact of obesity on invasive liver cancer. *Nutr Cancer*. 2017;69(8):1272-1280. doi:10.1080/01635581.2017.1367935.

72. Price RS, Cavazos D a, De Angel RE, Hursting SD, deGraffenried L a. Obesity-related systemic factors promote an invasive phenotype in prostate cancer cells. *Prostate Cancer Prostatic Dis*. 2012;15(2):135-143. doi:10.1038/pcan.2011.54.

73. Baldoli E, Maier JAM. Silencing TRPM7 mimics the effects of magnesium deficiency in human microvascular endothelial cells. *Angiogenesis*. 2012;15(1):47-57. doi:10.1007/s10456-011-9242-0.

74. Tharmalingam S, Daulat AM, Antflick JE, et al. Calcium-sensing receptor modulates cell adhesion and migration via integrins. *J Biol Chem*. 2011;286(47):40922-40933. doi:10.1074/jbc.M111.265454.

75. De Pergola G, Silvestris F. Obesity as a major risk factor for cancer. *J Obes*. 2013;2013. doi:10.1155/2013/291546.

76. Chiche J, Brahimi-Horn MC, Pouysségur J. Tumour hypoxia induces a metabolic shift causing acidosis: A common feature in cancer. *J Cell Mol Med*. 2010;14(4):771-794. doi:10.1111/j.1582-4934.2009.00994.x.

77. Higgins LH, Withers HG, Garbens A, et al. Hypoxia and the metabolic phenotype of prostate cancer cells. *Biochim Biophys Acta - Bioenerg*. 2009;1787(12):1433-1443. doi:10.1016/j.bbabio.2009.06.003.

78. Colella M, Gerbino A, Hofer AM, Curci S. Recent advances in understanding the extracellular calcium-sensing receptor. *F1000Research*. 2016;5:2535. doi:10.12688/f1000research.8963.1.

79. Muz B, Puente P de la, Azab F, Azab AK. The role of hypoxia in cancer progression angiogenesis metastasis and resistane to therapy. *Hypoxia*. 2015;3:83-92. doi:http://dx.doi.org/10.2147/HP.S93413.

80. Wojtkowiak JW, Rothberg JM, Kumar V, et al., Chronic autophagy is a cellular adaptation to tumor acidic pH microenvironments. *Cancer Res.* 2012;72(16):3938-3947. doi:10.1158/0008-5472.CAN-11-3881

81. Herroon MK, Rajagurubandara E, Hardaway AL, et al. Bone marrow adipocytes promote tumor growth in bone via FABP4-dependent mechanisms. *Oncotarget*. 2013;4(11):2108-2123. doi:10.18632/oncotarget.1482.

82. Bravo-Sagua R, Mattar P, D??az X, Lavandero S, Cifuentes M. Calcium sensing receptor as a novel mediator of adipose tissue dysfunction: mechanisms and potential clinical implications. *Front Physiol*. 2016;7(9):1-7. doi:10.3389/fphys.2016.00395.

83. Zhang C, Zhang T, Zou J, et al. Structural basis for regulation of human calcium-sensing receptor by magnesium ions and an unexpected tryptophan derivative co-agonist. *Sci Adv*. 2016;2(5). doi:10.1126/sciadv.1600241.

84. Grant CM, Kyprianou N. Epithelial mesenchymal transition (EMT) in prostate growth and tumor progression. *Transl Androl Urol*. 2013;2(4):202-211. doi:10.3978/j.issn.2223-4683.2013.09.04.