A ROLE FOR TRPV6 ION CHANNELS IN PROSTATE CANCER BONE METASTASES

A Dissertation in Anatomy

by

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ABSTRACT

Prostate cancer is the most common disease and second leading cause of cancer related deaths in North American men. In 2011, the National Cancer Institute estimated 240,890 new cases and 33,720 reported deaths related to prostate cancer in the United States. The financial burden of prostate cancer on the American public was estimated to be $9.9 billion in 2006 and is expected to grow as population’s age, cancer survival improves, and treatments become more costly. About 60-85% of men with advanced prostate cancer have associated bone metastases. Bone metastases contribute to bone pain and an increased risk of fracture which can potentially lead to paralysis due to spinal cord compression.

There are currently no effective therapeutic treatments to prevent prostate cancer bone metastases. Identifying mechanisms that facilitate favorable, early, prostate cancer cell to bone interactions will aid in the development of therapies to prevent bone metastases. The objective of this study was to identify a role for calcium and Transient Receptor Potential Vanilloid 6 (TRPV6) ion channels in the metastatic potential and early colonization of osteoblastic prostate cancer cells to murine bone. We proposed that transient increases in serum calcium, following parathyroid hormone 1-34 administration, confers a metastatic advantage to prostate cancer cells in circulation, signaled in part via TRPV6 ion channels.

We tested our central hypothesis by addressing the following specific aims: 1) Identify the effect of transient calcium elevations on osteoblastic prostate cancer cell metastatic potential, in vitro 2) Identify the requirement for TRPV6 expression in osteoblastic prostate cancer cell metastatic potential, in vitro. 3) Identify a role for TRPV6 expression in early prostate cancer cell colonization of murine bone, in vivo.

To identify the effect of transient calcium elevations on osteoblastic prostate cancer cell metastatic potential, we employed heterotypic cell adhesion, transwell migration, and
invasion assays. Observations from heterotypic cell adhesion assays suggested that C4-2 and C42B4 cells increased adhesion to hbmE cells pre-treated with extracellular calcium, 78% and 59%, respectively. Further, studies suggest that increased extracellular calcium enhanced E-Selectin, VCAM-1 and ICAM-1 cell surface abundance on hbmE cells. Moreover, studies with function blocking antibodies suggested these molecules were partially responsible for increased heterotypic cell adhesion. Finally, increased extracellular calcium enhanced migration of osteoblastic prostate cancer cell lines, *in vitro*. Together, observations from these studies suggested that increases in extracellular calcium enhanced the metastatic potential of osteoblastic prostate cancer cells, *in vitro*, and may serve an important role in modulating osteoblastic prostate cancer cell invasion and adhesion to bone marrow endothelial cells, *in vivo*.

To identify the requirement for TRPV6 expression in osteoblastic prostate cancer cell metastatic potential, we employed shRNA lentiviral particles to reduce TRPV6 expression in osteoblastic prostate cancer cell lines. Knockdown of TRPV6 expression was evaluated by quantitative PCR, western blot analysis, and immunocytochemistry. Proliferation, transwell invasion, and heterotypic cell adhesion assays were employed to evaluate metastatic potential, *in vitro*. Several clones were identified to have reduced TRPV6 mRNA and protein expression, ranging from 44-63%. Confocal micrographs of cells probed with antibodies specific to TRPV6 suggested TRPV6 cell surface localization in knockdown clones was also reduced. Prostate cancer cells with reduced levels of TRPV6 expression demonstrated slower proliferation rates and an impaired ability to adhere to and invade human bone marrow endothelial cells. Our observations support our hypothesis and suggest that TRPV6 has a role in modulating metastatic prostate cancer metastatic potential, *in vitro*. 
Lastly, to identify a role for TRPV6 expression in early prostate cancer cell colonization of murine bone, SCID/Beige mice were pre-administered parathyroid hormone 1-34 or vehicle intermittently, then inoculated with prostate cancer cells engineered to express reduced levels of TRPV6. Intermittent parathyroid hormone 1-34 administration yields transient increases in serum calcium, \textit{in vivo}. Eight weeks post prostate cancer cell inoculation, serum was collected and long bones and lumbar vertebrae were harvested for analysis. Bone tissue sections were probed with antibodies specific to prostate specific antigen and cytokeratin. Prostate specific antigen and cytokeratin positive cells were identified in long bones of 100\% of animals administered parathyroid hormone 1-34, relative to 20\% of animals treated with vehicle. Similar observations were made for animals injected with prostate cancer cells transfected with control shRNA lentiviral particles. In contrast, prostate specific antigen and cytokeratin positive cells were identified in only 20\% of the long bones of animals administered parathyroid hormone 1-34, and then inoculated with prostate cancer cells with reduced TRPV6 expression; however, there were no observed differences in the lumbar vertebrae. These observations suggest that a TRPV6 ion channels may have a role in PCa cell colonization to murine bone.

In summary, we have identified calcium and TRPV6 as potential mechanisms that facilitate favorable, early, prostate cancer cell to bone interactions, \textit{in vitro}. This is the first report employing shRNA to stably knockdown TRPV6 in a human osteoblastic prostate cancer cell line. This study provides evidence to suggest that \textit{in vivo}, changes in calcium concentrations in the bone environment may act upon bone marrow endothelial cells enhancing prostate cancer cell colonization to bone. We also provide evidence to suggest that TRPV6 has a role in modulating the interaction between osteoblastic prostate cancer cells
and bone marrow endothelial cells. The interaction between prostate cancer cells and bone marrow endothelial cells should be targeted in future studies to prevent bone metastases.
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LIST OF ABBREVIATIONS

1,25(OH)\textsubscript{2}D\textsubscript{3}: 1,25 dihydroxyvitamin D3
ALP: alkaline phosphatase
ATP: adenosine Triphosphate
BMD: Bone mineral density
BRC: bone remodeling compartment
CaCl\textsubscript{2}: calcium chloride
CaSR: calcium sensing receptor
EDTA: ethylenediaminetetraacetic acid
E-Selectin: endothelial-selectin
FBS: fetal bovine serum
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GFP: green fluorescent protein
GST: Glutathione S-transferase
H&E: hematoxylin and eosin
HEK-293: human embryonic kidney 293 cells
HFOB: human fetal osteoblast progenitor cells
hbmE: human bone marrow endothelial cells
HUVEC: human umbilical vein endothelial cells
HV: High voltage

ICAM-1: inter-cellular adhesion molecule
IP3R: inositol trisphosphate receptor Ca\textsuperscript{2+} release channel
IOD: Integrated optical density
μCT: microcomputed tomography
PBS: phosphate buffered saline
PCa: prostate cancer
PI3K: phosphoinositide 3-kinase
PIP2: phosphatidylinositol 4,5 bisphosphate
PKC: protein kinase C
PSA: prostate specific antigen
PTH: parathyroid hormone
qPCR: quantitative polymerase chain reaction
SD: standard deviation
SDS: sodium dodecyl sulfate polyacrylamide
TRAP: tartrate-resistant acid phosphatase
TRPV6: transient receptor potential vanilloid 6
VCAM-1: vascular cell adhesion molecule-1
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Chapter 1:

Introduction
Prostate Cancer, Calcium, and TRPV6 Ion Channels
Prostate cancer (PCa) is the most common disease and second leading cause of cancer related deaths in North American men [1-3]. In 2011, the National Cancer Institute estimated 240,890 new cases and 33,720 reported deaths related to PCa in the United States. Age is the most critical risk factor for the development of PCa; throughout the aging process, PCa risk increases from <30% in men over 50 years of age to 80% by age 80 [4, 5]. About 60-85% of men with advanced PCa have associated bone metastases [6]. PCa bone metastases contribute to bone pain and an increased risk of fracture which can potentially lead to paralysis due to spinal cord compression [7]. There are currently no effective therapeutic treatments to prevent or fully eradicate PCa bone metastases [1, 8]. A better understanding of how PCa cells colonize bone is needed to develop novel approaches towards therapeutic treatments that prevent skeletal metastases.

1.1 Prostate Anatomy and Function

The prostate is the largest accessory gland found in males and is about the size of a walnut, is located in the pelvis inferior to the bladder, and surrounds the male prostatic urethra [9]. McNeal identified three distinct anatomical regions within the prostate based on their relationship to prostatic disease: the peripheral zone, the transitional zone, and the central zone [10]. Benign prostatic hyperplasia, an enlarged prostate commonly seen in older men, occurs mainly in the transitional zone, while 70% of tumors occur in the peripheral zone [9]. The prostate is part of the reproductive system and produces 20-30% of the volume of semen. These secretions are rich in acid phosphatase which confers a protective effect for sperm. Prostate specific antigen (PSA) is also secreted from the prostate which is a glycoprotein that acts to liquefy the semen. Additionally, prostatic secretions contain sugars, sulfate, and Vitamin E which prevents clumping of the sperm [11].
1.2 Trends of Prostate Cancer

PCa is rarely diagnosed in men under the age of 40, more than 70% of patients diagnosed with PCa are between the ages of 60-80 years [12]. It is estimated that 15-30% of men over 50 and 80% of men over 80 years have undiagnosed PCa [13]. A temporal study in the US, reported that 98% of men diagnosed with PCa between 2001-2006 were between the ages of 40-49 years [14]; although, the majority of these men (95%) had low risk PCa, defined as moderately or poorly undifferentiated localized cancer. Geographic distribution of fatal PCa indicates that there are higher mortality rates in populations living at northern latitudes and among African-American men [14, 15]. It is not clear why this disparity exists but it is has been speculated that African-American men are at a genetic risk for developing PCa [16]. The lowest number of cases reported is among Japanese and Indian men not living in the U.S. [17]. There is also a 10 fold increase in the incidence of clinically significant PCa in Western industrialized countries compared to East Asian countries suggesting lifestyle as a risk factor [17, 18]. Additionally, individuals who consume a diet high in fat, dairy, and/or red meat are thought to be at a higher risk of developing PCa [18, 19].

1.3 Prostate Cancer Diagnosis

PCa is usually diagnosed by an abnormal digital rectal exam and/or elevated PSA serum levels. A malignant prostate often feels hard and irregular upon physical examination [20]. If an irregular prostate is suspected, PSA serum levels will be measured. PSA is secreted from a health prostate at very low levels but increases in the presence of PCa [13]. PSA levels of \( \leq 2.5 \) ng/ml are considered normal, PSA levels tend to fluctuate and when PSA levels continually rise over a period of time PCa is suspected [21]. A prostate biopsy is the only test that can confirm the presence PCa. Biopsy results are reported using a Gleason
score which indicates how aggressive the PCa is suspected to be on a scale from 2-10. The higher the Gleason score the more likely it is that the PCa has spread to areas outside of the prostate, for example a Gleason score of < 7 is considered to be low grade PCa [22]. Radiographic imaging or radionuclide bone scans are often performed to determine whether PCa has metastasized to other locations [23]. The results of these tests will then determine how physicians proceed in the treatment of PCa.

1.4 Prostate Cancer Treatment

There are several treatment options available to treat PCa and are dependent on the severity of the cancer at the time of diagnosis. The standard treatment for localized PCa is a radical prostatectomy, removal of the prostate [24]. Removal of the prostate while effective in treating localized PCa, can result in urinary incontinence and erectile dysfunction [25]. Androgen deprivation is commonly used to treat advanced PCa [24]. The prostate relies on androgens to function properly. It has been well demonstrated that androgen ablation therapy induces a rapid apoptotic response in prostate epithelial cells [26]. Androgen deprivation therapy is characterized by an orchiectomy, removal of the testes, and/or administration of gonadotropin releasing hormone agonists [27]. Radiation therapy is performed when it is thought that PCa has not spread from the prostate and is commonly used in conjunction with a radical prostatectomy and/or androgen deprivation. In some cases when treatment could complicate other diseases, physicians will choose to monitor the disease by regular PSA serum screenings. Chemotherapy is usually done when PCa is no longer responsive to hormone treatment. Many more treatment options are available to treat PCa but unfortunately they are only effective when the cancer is caught early [22].
1.5 Challenges of Treatment

One challenge when treating localized PCa is to distinguish between patients with indolent or aggressive PCa. There is a need for markers which identify PCa behavior to assist patients with diagnosed low risk PCa in making a decision to seek invasive treatment or continue with watchful waiting [28]. Most men with detected high levels of PSA will elect to receive some form of treatment [29], although, PSA is not a fail proof test with an absolute value distinguishing cancer from non-cancer [30]. Further, the correlation between PSA serum levels and PCa has dropped from 0.7 to 0.1 because of over screening and treating patients with low risk PCa defined as a Gleason score of $\leq 6$ [31]. It has been suggested that PCa is over-detected and thus over-treated in men over the age of 70 by 77% [31]. According to Thompson et al., upon diagnosis of PCa, regardless of Gleason score, there is a 14% chance of succumbing to the disease [32]. The risks of androgen deprivation therapy on patients with PCa create a necessity for the differentiation between non-invasive and aggressive PCa.

Androgen deprivation is accompanied by several adverse effects including diabetes, cardiovascular disease, fracture, and osteoporosis [27]. Androgen deprivation leads to an increase in bone resorption which decreases bone mineral density (BMD) and increases the risk of fractures and osteoporosis [33]. These factors can be attributed to a reduction of estrogen, contributing to a decline in the maintenance of skeletal mass [24]. One study reported that 18.4% of 81,000 men treated for PCa by androgen deprivation therapy experienced at least one fracture within the 3 years after treatment [33].

Despite efforts toward early detection of localized PCa, there is a 10% probability that some men already have micro metastases at the time of diagnosis [34]. Furthermore, it is
estimated that 20-40% of men previously treated for PCa will have recurrent elevated PSA levels indicative of recurrent PCa [2]. Recurrent PCa usually progresses from androgen dependent to androgen independent over the course of treatment which is a more aggressive form of PCa [1, 8]. Following this transition, chemotherapy is rendered ineffective because androgen independent PCa has a slower rate of proliferation [35]. Once PCa has become androgen independent the median survival rate is 20-24 months [36]. The risk of developing bone metastases once PCa has become androgen independent is significantly increased; it is estimated that 65-75% of these patients will develop bone metastases [37]. Unfortunately, there are no therapeutic treatments available to fully eradicate PCa bone metastases [1, 8]. It is therefore essential to identify cellular mechanisms necessary for the early colonization and establishment of PCa metastases to bone in order to develop novel preventative measures and treatments.

1.6 Healthcare Costs associated with Prostate Cancer

The financial burden of cancer on the American public was estimated to be $104.1 billion in 2006 and is expected to grow as US populations age, cancer survival improves, and treatments become more costly. PCa treatment costs are ranked fifth in the country, behind breast cancer, colorectal cancer, lung and lymphoma, at an estimated $9.9 billion [38]. There is increasing evidence that health care costs are rising as newer technologies are developed for treating PCa [39]. One study compared traditional treatment methods of radical prostatectomy surgery and three dimensional conformal radiation therapy to more costly alternatives such as robotic assisted surgery and intensity modulated radiation therapy and found that since 2005 when these methods became popular it has increased national spending by more than $350 million [39]. Furthermore, androgen deprivation therapy has associated
co-morbidities such as increased risk of osteoporosis and fracture. Patients treated for PCa without electing androgen deprivation therapy spend approximately $29,044 over a 3 year period. Of the patients who elected androgen deprivation therapy, 18% sustained skeletal fractures which brought the 3 year total health care cost to $48,350 per patient [40]. As health care costs continue to grow, it is imperative to determine the appropriate treatment methods for patients with PCa based on their risks of developing advanced metastatic disease.

1.7 Characteristics of Metastatic Prostate Cancer

In spite of rigorous research, little is known about the mechanism of how PCa cells colonize and establish lesions in bone. About 90% of PCa skeletal metastases are osteoblastic (bone forming) in nature unlike other cancers that metastasize to bone and form osteolytic (bone resorbing) lesions such as breast cancer [41-44], which suggests that bone turnover/formation may have an important role in PCa cell colonization. Proposed theories of primary tumor cell spread to distant secondary sites include venous drainage, lymphatic circulation, and arterial emboli [41, 45-47]. In 1889, Paget developed his “seed and soil” hypothesis that postulated an extravasated cancer cell “the seed” is only compatible with certain microenvironments “the soil” [48]. Ewing challenged Paget’s theory and suggested that disseminated tumor cells formed metastases because of mechanical factors such as being trapped in capillary beds [46]. This theory does not however explain why metastases appear in organs that do not have a large blood supply [49]. In support of Ewing’s theory, Batson observed that veins draining the prostate first encountered a capillary bed in the lumbar spine, a common site for PCa bone metastases [45, 50]. In addition, Fidler et al. determined that although tumor cells do indeed get lodged in capillary beds, less than 0.01% survive long enough to develop metastases [51]. It is therefore important to consider that although tumor
cells can be found in capillary beds they do not always lead to metastases. Nonetheless, it appears that certain cancers have a preferential ability to metastasize to certain organs while others do not, this preference results from a combination of mechanical and “soil” factors [49].

Common skeletal sites of PCa bone metastases, listed in order of frequency, include the spine, femur, pelvis, rib cage, sternum, humerus, and skull, all sites with elevated rates of bone turnover [41, 52, 53]. Several groups have indicated that PCa preferentially localizes to areas of high bone turnover [53-55]. Osteoblastic lesions commonly appear as woven bone on existing trabeculae [56]. Woven bone is less structurally sound as it is a collection of highly mineralized randomly oriented collagen fibrils [7]. This suggests that PCa cells induce de novo bone formation [43]. For instance, Correy et al showed that intratibial xenografts of PCa cells induced bone formation [57], however, de novo bone formation could have resulted from needle placement into the shaft of the tibia to facilitate cell placement. Moreover, serum levels of bone specific alkaline phosphatase, a marker of osteoblast activity, increase in PCa patients with bone metastases, compared to patients without bone metastases [58-60]. It has been recommended to include markers of osteoclast and osteoblast activity as prognostic tools for PCa as there appears to be a positive relationship between increased serum markers of bone turnover and PCa progression [61]. Further research needs to be done to determine the specific cellular mechanisms required for PCa cells to establish lesions within the bone environment.
1.8 Prostate Cancer Models

1.8.1 Cell Lines

A variety of PCa cell lines are available to investigate the mechanisms involved in PCa progression. The most commonly used PCa cell lines are LNCaP, PC-3, and DU-145. The LNCaP PCa cell line was isolated from a human metastatic lymph node and is considered an androgen dependent nonmetastatic cell line [62]. Wu et al., further developed C4 and C5 sub-lines from the LNCaP cell line to mimic the progression of PCa from androgen dependent to androgen independent [63]. A human fibroblast cell line, MS, isolated from a patient with osteogenic sarcoma, and LNCaP cells were co-injected subcutaneously into male athymic mice and after 8 weeks, mice were castrated. The C4 and C5 LNCaP sub-lines were isolated from the castrated mice 4 and 5 weeks after castration, respectively. C4 and MS cells were then subcutaneously co-injected into castrated athymic mice and cells were isolated from subcutaneous tumors 12 weeks after injection creating the second generation PCa cell line, C4-2. These cells are androgen independent and osteoblastic in nature. The C4-2B4 cell line is a derivative of the C4-2 cell line which was isolated from a metastatic PCa lesion found in the lumbar spine of a castrated athymic mouse. This cell line is androgen independent, considered highly aggressive, and forms osteoblastic lesions [64].

PC-3 and DU-145 PCa cell lines were established from human metastatic PCa isolated from the bone and brain, respectively, these cell lines are also androgen independent [65, 66]. While these cell lines are commonly used because they aggressively home to bone [64], they do not exhibit characteristics commonly associated with human PCa metastases; for example, they form primarily osteolytic bone lesions which are more arguably characteristic of breast cancer metastases [67]. The advantage to using the LNCaP derived PCa cell lines C4-2 and
C4-2B4 is that they mimic the progression of the human disease by spontaneously producing osteoblastic bone lesions. For a detailed account of available PCa cell lines please refer to Webber [68].

1.8.2 Animal Models Used to Study Metastasis

Currently, there are few animal models available to study the progression of PCa. There are studies that have simulated PCa progression from androgen dependence to androgen independence by castrating male mice and allowing the tumors to adapt, this model closely mimics the human disease [63]. In some cases, LNCaP sub-lines will spontaneously metastasize to bone but this process requires 4-6 months for establishment with an overall colonization rate between 15-20% of all animals developing bone metastases [69]. Other, more efficient models to study bone metastases include direct interosseous injection into mouse long bones [64, 70], direct injection of PCa cells into human fetal bone xenografts [71], and surgical orthotopic implantation of tumor pieces into male mouse prostates [72]. Direct interosseous injections of PCa cells damages the bone and releases factors which may not be present otherwise that could facilitate the establishment of bone lesions [57]. There is a higher incidence of observed bone tumors in the human fetal bone xenograft model, however, the use of fetal bone complicates the metastatic process because of the presence of bone derived factors not present in adult bone [71]. Surgical orthotopic implantations do result in metastatic bone lesions, however, there is higher rate of metastatic lesions that develop in the liver, brain, and kidney that are not observed in the human disease [72]. While these models can be used to study the interaction of PCa cells with bone, they are less effective for the study of early metastatic events in bone. For a more in depth review of the animal models discussed above please see DeGraff et al. [73]. Other studies suggest model
systems employing the administration of human parathyroid hormone (PTH) may be useful for identifying mechanisms of PCa colonization of bone [53, 74]. Our laboratory observed an increase in PCa cell uptake and colonization of murine bone in animals administered PTH [74].

1.9 Parathyroid Hormone

PTH is an 84 amino acid polypeptide that has a central role in maintaining calcium homeostasis. It is secreted by parathyroid glands in response to low serum calcium levels and acts to stimulate calcium reabsorption from the kidney, enhance calcium absorption from the intestine, and promote bone resorption to increase serum calcium [11]. Exogenous PTH, known as teriparatide, has commonly been used for the treatment of osteoporosis [75]. Teriparatide contains the first 34 amino acids of PTH (PTH1-34) and is sufficient for its biological activity [76].

With regard to action on bone, PTH has both anabolic (bone forming) and catabolic (bone resorbing) effects. When administered continuously, PTH enhances bone resorption. As Iida-Klein and colleagues demonstrated, two weeks of continuous PTH infused subcutaneously into female C57BL/J6 mice increased osteoclast number, decreased BMD, but did not significantly change osteoblast activity [77]. In contrast, intermittent PTH administration promotes bone formation, marked by increased bone mass and enhanced mechanical properties of bone [78]. PTH administration has recently become a staple in the treatment of osteoporosis. Studies suggest that intermittent PTH administration increases bone formation largely by enhancing the number and activity of osteoblasts by increasing osteoblastogenesis, attenuating osteoblast apoptosis, and/or activating bone lining cells [79-84]. Studies within our own laboratory suggested that PTH had varying effects on bone
formation depending on the dose; in one study, 40 µg/kg significantly enhanced bone turnover as well as bone formation [74] while another study found that 20 µg/kg was sufficient to enhance bone formation [85].

1.10 Parathyroid hormone and PCa Bone Metastases

PCa animal model studies involving PTH are limited, but in one example, Schneider and colleagues demonstrated that by administering PTH 1-34 before and after PC-3 inoculation into the left ventricle of athymic mice, an increased number of osteolytic metastases formed compared to mice treated with saline [53]. Our lab has also demonstrated that intermittent PTH injections significantly enhanced the colonization of C4-2 cells to murine bone. C.B-17 SCID/Beige mice were administered intermittent PTH 1-34 injections [40 µg/kg] over a period of three weeks. Intermittent is defined as one injection daily for 5 days followed by 2 days without an injection. C4-2 cells were delivered into animals by intracardiac injections and a cohort of animals received an additional cycle of intermittent PTH injections. PSA serum analysis and immunohistochemistry indicated that 70% of the animals had clusters of PCa cells within the bone marrow and trabecular spaces of long bones [74]. These two studies suggest that PTH enhances the colonization of PCa cells to murine bone, in further support these observations, the administration of PTH for the treatment of osteoporosis has been correlated with the development of osteosarcoma, a rare bone cancer [86-88]. The specific cellular mechanisms involved in this process, however, remain elusive.

Endogenous PTH serum levels may be correlated to PCa progression. Patients with advanced PCa, those with or without associated bone metastases, have been reported to have increased serum levels of PTH [89-92]. In a study by Murray et al, 88% of patients with
diagnosed PCa had increased serum levels of PTH compared to age matched controls; this included patients with no bone involvement as well as patients with established bone metastases [92]. Additionally, Buchs et al. reported 69% of patients with bone metastases had increased PTH serum levels [42]. In one epidemiologic study, serum PTH and serum calcium were positivity correlated to PSA levels, a marker for PCa, in men with diagnosed PCa [93]. We propose that serum calcium levels, regulated by PTH, are involved in the progression of PCa bone metastases.

1.11 Serum Calcium and Prostate Cancer

Interestingly, serum calcium is known to transiently increase in response to a single injection of PTH in humans and rodents [94-96]. In one study, a single dose of PTH 1-34 (20 µg) transiently increased serum ionized calcium (~0.92 mmol/L) within 2-4 hrs after administration. Serum calcium returned to basal levels (1.23 ± 0.03 mmol/L) within 24 hrs after treatment [96]. Recently, increased basal serum calcium levels have been linked to a risk of developing fatal PCa [93, 97, 98]. Studies conducted by Skinner et al. found that high pre-diagnostic serum calcium levels (2.36-3.06 mmol/L) compared to low pre-diagnostic serum calcium levels (1.06-2.27 mmol/L), measured an average of 9.9 years prior to diagnosis, were correlated with a higher risk of developing fatal PCa [93, 97]. It is important to specify that serum calcium levels were not measured at the same time every day and that serum calcium levels have a circadian variation which is highest in the early morning and evening [99, 100]. Therefore, these reports of elevated serum calcium levels may not be sustained elevations and could be transient elevations. It has also been suggested that a dietary intake of calcium exceeding 1500-2000 mg/day is correlated to an increased risk of developing advanced metastatic PCa [101-103]. These studies suggest a greater role of serum
calcium in the development and advancement of PCa than previously thought. We propose that transient increases in serum calcium, following rises in PTH, offer a survival advantage to PCa cells within circulation.

1.12 Biological Function of Calcium

Calcium ions are essential in regulating whole body homeostasis and play a vital role in cellular functions including proliferation, differentiation, and apoptosis [104]. Most calcium is obtained through diet and absorbed through the intestine [34]. Ionized calcium levels in healthy individuals range from 0.47-0.55 mmol/L [6, 52]. Calcium homeostasis is modulated by many different factors including PTH and 1,25 dihydroxyvitamin D3 (1,25(OH)2D3). When serum calcium levels fall, the parathyroid gland senses this decrease and releases PTH which acts on the kidney and the bone to increase serum calcium levels. In the kidney, PTH activates calcium reabsorption and hydroxylation of 25-hydroxyvitamin D to the metabolically active form, 1,25(OH)2D3 [105]. Under physiological conditions, high levels of PTH are associated with increased bone resorption and subsequently increased serum calcium. During bone turnover, calcium is released or incorporated into the bone matrix [52, 106, 107]. During bone resorption extracellular calcium levels within resorbed lacunae can reach levels between 8-40 mmol/L [52, 108]. Our study further investigates the role that calcium has in the colonization of osteoblastic PCa cells.

1.13 TRPV6 Ion Channels

1.13.1 Introduction of TRPV6 Ion Channels

Transport of calcium across epithelial plasma membranes is mediated by a superfamily of ion channels, transient receptor potential (TRP) channels. In addition to epithelial calcium transport, this family of 20 cation channels has a variety of other
physiological functions including phototransduction, nociception, olfaction, and hot and cold sensation [109]. TRPV6 is a member of the TRP vanilloid (V) subfamily and is predominantly expressed in epithelia involved in calcium absorption such as the small intestine, kidney, and placenta [110]. TRPV6, originally discovered and cloned from the rat duodenum [111], originates on human chromosome 7q33-q34 [112]. TRPV6 consists of six transmembrane segments with intracellular amino and carboxy terminal ends. TRPV6 is known to form heterotetramers creating a hydrophobic pore region between segments 5 and 6. TRPV6 is highly sensitive and selective for calcium ions and under physiological conditions conducts only calcium ions but is permeable by other divalent cations [107, 113, 114].

1.13.2 Regulation of TRPV6 Ion Channel Activity

TRPV6 channel activity, opening and closing of the pore, is regulated by several mechanisms. The S100A10-annexin-2 complex and rab11a have been reported to regulate TRPV6 localization to the plasma membrane in human embryonic kidney 293 (HEK-293) cells [115, 116]. TRPV6 ion channels in HEK-293 cells have also been reported to be trapped in the plasma membrane by klotho, a β-glucuronidase, through hydroxylation of extracellular sugar residues [117]. Binding of phosphatidylinositol 4, 5 bisphosphate (PIP2) has been demonstrated to activate TRPV6 ion channels [118]. Calmodulin binding to the C-terminal tail of TRPV6 has been shown to contribute to channel inactivation when intracellular calcium levels rise; phosphorylation of protein kinase C (PKC) has been reported to block this inactivation by preventing calmodulin from binding [119]. TRPV6 is also activated by low intracellular calcium through the inositol trisphosphate receptor Ca2+ release (IP3R) channel. Activation of IP3R channels on the endoplasmic reticulum
inactivates TRPV6 by increasing intracellular calcium levels [120]. Studies have indicated that TRPV6 channel expression is regulated by 1,25(OH)_{2}D_{3} [110, 114, 119, 121]. Taparia and colleagues demonstrated in Caco-2 colon cancer cells that regulation of TRPV6 by 1,25(OH)_{2}D_{3} requires mRNA synthesis as opposed to protein modification [122]. Estrogen and androgens have also been reported to regulate TRPV6 channel expression [107, 122-125]. The redundancies of mechanisms that regulate TRPV6 activity suggest that this channel may be important in cellular function and make it an interesting therapeutic target.

1.13.3 TRPV6 Ion Channels and PCa

Recently, TRPV6 was found to be highly expressed in localized PCa [107]. More importantly, TRPV6 ion channel expression is undetectable in nonmalignant prostate tissue [113]. Fixemer et al. analyzed prostate tissue biopsy samples from locally advanced tumors and lymph node metastases and found that TRPV6 channel expression positively correlated with Gleason scores [126]. The function of TRPV6 in non-metastatic PCa cell lines has been studied and although the mechanisms have not been fully investigated, TRPV6 seems to play a role in PCa cell proliferation and survival. Lehen’kyi et al. used siRNA to reduce TRPV6 in LNCaP cells and found that proliferation was decreased and the percent of apoptotic cells was increased [127]. A role for TRPV6 in the establishment of PCa bone metastases has yet to be investigated.

1.13.4 Possible Cellular Signaling Mechanism Regulating TRPV6 Ion Channels

The predominant cell surface sensor for extracellular calcium is the calcium sensing receptor (CaSR), a heterotrimeric G-protein–coupled receptor [128]. In one study, CaSR expression was ablated by RNA interference in PC-3 PCa cells which were injected into the left ventricle of athymic mice. Reduced CaSR expression suppressed the development of
bone metastases compared to normal PC-3 cells [52]. It has not been reported that the CaSR regulates TRPV6 expression, however, multiple studies have suggested TRPV6 is a receptor operated channel [129]. TRPV6 is activated by the androgen, estrogen, and the prostate specific G-protein-coupled receptors [125, 127, 129]. It is well understood that activation of a G-protein coupled receptor (i.e. CaSR) leads to downstream activation of the PI3K/AKT signaling pathway. Further, it has been reported that PI3K increases intracellular calcium movement via TRPV6 [130]. Within this study we provide evidence to suggest that the CaSR and PI3K may be linked to TRPV6 ion channel expression.

1.14 Interactions between PCa cells and Endothelium

1.14.1 The Bone Remodeling Compartment

Evidence suggests that changes in metabolic bone activity are involved in the development of PCa bone metastases [53-55]. There is limited information available as to which particular aspect of bone activity is necessary for the colonization of PCa cells to bone, whether it be bone formation, bone resorption or both. Bone remodeling is a tightly regulated process that includes combined actions of osteoblasts, osteoclasts, and osteocytes. During early colonization, one route through which circulating PCa cells could reach the bone microenvironment is the Bone Remodeling Compartment (BRC). The BRC is an area where cancellous bone resorption and formation occur concurrently under a canopy of bone lining cells and adjacent sinusoids, physically separated from the bone marrow by flattened cells [131]. After bone resorption is initiated, bone lining cells on the quiescent surface rise to form a roof over remodeling cancellous bone, forming the BRC. The BRC was identified in tissue sections taken from bone biopsies of patients who had primary hyperparathyroidism (excess levels of PTH). Samples were taken prior to parathyroidectomy and three years after
surgery. Comparisons of pre and post-surgery tissue sections, indicated that patients with primary hyperparathyroidism, known to have increased bone resorption, displayed a significantly increased number of BRCs [131]. There are reports of patients with advanced PCa, with or without associated bone metastases, with increased serum levels of PTH [89-92]; we speculate that they also have an increased number of BRCs. While it is postulated that the BRC may play a crucial role in the spread of bone metastases, no studies have been completed to confirm this hypothesis [132]. Erythrocytes are commonly seen within this sinus suggesting a circulatory function of the BRC [133]. Additionally vascular elements have been reported to be located adjacent to the bone lining cells forming the canopy of the BRC [133]. This compartment provides an opportunity for PCa cells to enter the bone microenvironment through adjacent sinusoids.

1.14.2 Interaction between PCa cells and Bone Marrow Endothelial Cells

Within our model, PCa cells from the circulation would have to first adhere to and then migrate through bone marrow endothelial cells prior to reaching the bone microenvironment. It has previously been shown that PCa cells, LNCaP and PC-3 cell lines, preferentially adhere to bone marrow endothelial cells compared to human umbilical vein endothelial cells (HUVEC) [134]. Endothelium serves as a barrier to regulate the passage of cells across the endothelial cell layer [135]. Adhesion molecules present on the cell surface of endothelial cells are responsible for selectivity and interact with ligands on the circulating cell’s surface [136]. During an inflammatory response, TNFα and IL-1 activate endothelial cells which increase the surface expression of several adhesion molecules including endothelial-selectin (E-selectin), inter-cellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells [137]. E-Selectin is responsible for
the initial tethering of leukocytes; this tethering then activates intracellular signals which increase the surface expression of leukocyte integrins which bind to ICAM-1 and VCAM-1. The cellular interaction of ICAM-1 and VCAM-1 with integrins on leukocytes firmly attaches them to endothelial cells and brings them into position for transendothelial migration [138]. It is, therefore, reasonable that metastatic PCa cells may use similar adhesion molecules to reach the bone microenvironment.

1.14.3 Adhesion Molecules Present on Bone Marrow Endothelial Cells

Cell adhesion molecules are composed of carbohydrates and are necessary for cell-cell and cell-matrix interactions required for cellular proliferation, differentiation, and tumor invasion and metastasis [139]. E-Selectin and VCAM-1 have been shown to be necessary for the adhesion of hematopoietic progenitor cells to bone marrow endothelial cells and subsequent migration to reach the marrow compartment in bone [140]. PC-3 and Du-145 PCa cells have been shown to preferentially bind to E-selectin, in vitro [141]. Upon activation by TNFα and IL-1, E-Selectin, ICAM-1, and VCAM-1 increase cell surface abundance within 4-6 hrs [142]. Further, it has been demonstrated that increases in intracellular calcium, induced by low density lipoprotein, increase expression of VCAM-1 and E-Selectin in human aortic endothelial cells [143]. This suggests that increases in extracellular calcium may also increase E-Selectin, ICAM-1, and VCAM-1 cell surface abundance; however, these studies have not been conducted.

1.15 Summary

There are currently no effective therapies available to fully eradicate PCa bone metastases despite advancements in early detection and treatment of PCa. To begin to develop therapies to treat PCa bone metastases a better understanding of the cellular
mechanisms required for PCa cell early colonization of bone is required. There are few investigations which seek to identify how PCa cells colonize bone. Our previous study suggested that increased bone formation facilitated the uptake and colonization of a PCa cell line that otherwise demonstrated a poor capacity to colonize bone when placed in circulation. The mechanism responsible for this enhanced colonization remains elusive and may be due to a combination of factors including an increase in the number of BRC’s, adhesion molecules, release of growth factors from remodeling bone, and/or serum calcium. It is known that serum calcium levels increase in response to PTH, which we propose is a possible cellular mechanism involved in the colonization of PCa cells to bone. Further, we suggest that increases in extracellular calcium are sensed by PCa cells in part through TRPV6 ion channels. This thesis identifies the role that calcium and TRPV6 ion channels have in the metastatic potential of osteoblastic PCa cells, in vitro and in vivo.
Chapter 2:

Transient Elevations in Calcium Enhance Adhesion and Invasion of Osteoblastic Prostate Cancer Cells
2.1 Abstract

We previously reported that intermittent PTH 1-34 administration enhanced the colonization of human osteoblastic prostate cancer cells to murine bone. While the mechanism is unknown, we propose that transient increases in serum calcium following PTH 1-34 administration confer a metastatic advantage to circulating PCa cells. We tested the hypothesis that increases in extracellular calcium enhance adhesion, invasion, and migration of osteoblastic PCa cells, \textit{in vitro}. To test our hypothesis, C4-2 and C4-2B4 osteoblastic PCa cell lines were used in heterotypic cell adhesion (C4-2 to human bone marrow endothelial (hbme) cells) assays, transwell migration assays and invasion assays. Interestingly, heterotypic cell adhesion assays demonstrated that C4-2 and C4-2B4 cells increased adhesion to hbme cells treated with CaCl$_2$ (1.25 mM) by 78% and 59%, respectively. Further studies suggested that CaCl$_2$ (1.25 mM) enhanced E-Selectin, VCAM-1 and ICAM-1 cell surface abundance on hbme cells and that these molecules were partially responsible for the increased in adhesion observed. We further demonstrated, by transwell migration assays, that increased extracellular calcium (2.5 mM CaCl$_2$) enhanced migration of osteoblastic PCa cell lines. Our observations supported our hypothesis and suggest, for the first time, that extracellular calcium may serve an important role in modulating metastatic PCa cell invasion and adhesion to bone marrow endothelial cells, \textit{in vitro}.

2.2 Introduction

Prostate cancer is the most common disease and second leading cause of cancer related deaths in North American men [1-3]. In 2011, the National Cancer Institute estimated 240,890 new cases and 33,720 reported deaths related to PCa in the United States. There are currently no effective therapeutic treatments to prevent PCa bone metastases. Identifying
mechanisms that facilitate favorable, early, PCa cell to bone interactions will aid in the development of therapies to prevent bone metastases. We have previously demonstrated that PTH 1-34 administration enhances the uptake and colonization of C4-2 PCa cells to murine bone [74]. Eight weeks following PCa cell inoculation, micrometastases were identified within the marrow spaces of long bones in 70% of the animals treated with PTH. While this is the first study to demonstrate colonization of osteoblastic PCa cells to murine bone in a period shorter than 4 months, the cellular processes that modulate this colonization need further investigation.

Herein, we propose that serum calcium is involved in the development of PCa bone metastases. Serum calcium levels transiently increase in response to a single injection of PTH [94, 95]. Recently, elevated serum calcium levels have been linked to a risk of developing fatal PCa; Skinner et al., found that elevated pre-diagnostic serum calcium levels were correlated with a higher risk of developing fatal PCa [2, 93, 97]. It has also been reported that a dietary intake of calcium exceeding 2,000 mg/day was correlated to an increased risk of developing advanced metastatic PCa [101, 102, 144]. Furthermore, during bone turnover, calcium levels within the proximity of resorbed lacunae can reach levels between 8-40 mmol/L [52, 108] which ultimately will increase serum calcium levels. Additionally, patients with advanced PCa, with or without associated bone metastases, have been reported to have increased serum levels of PTH [89-92, 141] which could potentially lead to transient increases in serum calcium. Within this study, we propose that elevations in extracellular calcium enhance osteoblastic PCa cell adhesion, invasion, and migration, \textit{in vitro}.

Calcium ions are essential in regulating whole body homeostasis and play a vital role in cellular functions including proliferation, differentiation, migration and apoptosis [104].
Several studies suggest that increases in extracellular calcium have a role in the development and progression of cancer, including breast, colorectal, and prostate [52, 145, 146]. Increased extracellular calcium, in vitro, enhances the adhesion and migration of thyroid carcinoma cells [128]. In another study, high levels of extracellular calcium were reported to increase the migration of metastatic breast cancer cell lines but not nonmetastatic cell lines [146]. Additionally, Liao et al., have reported that increased extracellular calcium enhances PC-3 proliferation and cellular attachment to tissue culture plastic, in vitro [52].

We tested the hypothesis that increased extracellular calcium enhances the adhesion, invasion, and migration of osteoblastic PCa cells, in vitro. Employing heterotypic cell adhesion assays, we demonstrated that C4-2 cell adhesion to hbmE cells is enhanced (77%) when hbmE cells are pretreated with CaCl$_2$. Western blotting and immunocytochemistry suggested that CaCl$_2$ increased the abundance of E-Selectin, ICAM-1, and VCAM-1 on the surface of hbmE cells. Function blocking antibodies suggested that these adhesion molecules are involved in the increased adhesion we observed. We also demonstrated that CaCl$_2$, acting as a chemoattractant, enhanced osteoblastic PCa cell migration and invasion of hbmE cell monolayers. These studies suggest that increases in extracellular calcium enhance the metastatic potential of osteoblastic PCa cells and provide evidence that calcium may significantly impact PCa cell uptake and colonization of bone.

2.3 Materials and Methods

2.3.1 Cell Culture

Human C4-2 and C4-2B4 PCa cell lines, kindly provided by Dr. Robert A. Sikes (University of Delaware), were cultured in T-Medium (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v)
penicillin/streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO₂. Cells were sub-cultured when 80-90% confluent using 0.05% (v/v) trypsin/ethlenediaminetetraacetic acid (EDTA). All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. All experiments were completed using cells between passages 13-18.

Human bone marrow endothelial cells (hbmE), provided by Dr. G Almeida-Porada (University of Nevada, Reno), were cultured in M199 medium supplemented with endothelial cell growth supplement (R&D systems Inc., Minneapolis, MN), 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin, 2 mM glutamx and heparin (15 U/ml). Cells were sub-cultured when 80-90% confluent using 0.05% (v/v) trypsin/EDTA. All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. All experiments were completed using cells between passages 5-12.

The human fibroblast cell line, C3H10T1/2, was cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin. Human fetal osteoblast progenitor cells (HFOB), provided by Dr. Henry Donahue, were also cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin. All cells were sub-cultured similarly. All experiments were completed using cells between passages 3-7.

2.3.2 Proliferation Assay

PCa cells (5,000) were seeded in 96 well plates in T-Medium (Invitrogen, Carlsbad, CA) in 1% (v/v) serum and allowed to adhere for 6 hrs. CaCl₂ [1.25 mM] was then added to cultures for the duration of the experiment (continuous) or for 3 hrs/day (transient) and the medium was replaced with T-Medium. Every 24 hrs, cell number was quantified using a
CyQuant Proliferation Assay (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol, or cell counts were performed using trypan blue exclusion.

2.3.3 Cell Viability Assay

To test the hypothesis that calcium enhances PCa cell survival, cell death was induced with serum starvation. C4-2 cells were seeded in 96 well plates (8,000/well), about 30% confluence, and on the day of the experiment, medium was changed to one of the following conditions: 1% (v/v) serum, positive control, or 0% (v/v) serum +/- 2.50 mM CaCl$_2$. CaCl$_2$ was either added for the duration of the experiment (continuous) or for 3 hrs/day (transient). After 72 hrs, the medium was removed and 0.5 mg/mL of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO) solution was added to each well to measure cellular activity. Following 4 hrs of incubation, at 37°C, 100 µl of DMSO was added to all wells to dissolve the crystals and the plate was read at 570 nm using a spectrophotometer.

Adenosine Triphosphate (ATP) was used as another method to induce cell death [147]. A range of ATP concentrations [0-3000 mM] were tested and 3,000 mM of ATP was identified as the concentration sufficient to decrease MTT activity. In subsequent experiments, 3,000 mM ATP +/- 2.50 mM CaCl$_2$ was added to the C4-2 cells for a period of 48 hrs. Cellular activity was assessed as discussed above.

2.3.4 Heterotypic Cell Adhesion Assay

To test the hypothesis that increases in extracellular calcium enhance PCa cell adhesion to hbmE cells, adhesion assays were performed. HbmE cells (80,000) were seeded in 48 well tissue culture dishes and allowed to become confluent monolayers. On the day of the experiment, hbmE monolayers or sub-confluent C4-2 cells were treated with 1.25 mM
CaCl₂ for 3 hrs. C4-2 cells were washed with phosphate buffered saline (PBS) then removed with 5 mM EDTA and labeled with 25 µM of Calcein-AM (Molecular Probes, Invitrogen, Carlsbad, CA) for 20 min. C4-2 cells were then washed two times with serum-free RPMI and placed on top of hbmE cell monolayers for a period of 3 hrs. Cells were then washed with PBS and gently agitated to remove non-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). Adhesion assays were repeated with C4-2B4 PCa cells to test another PCa cell line and HFOB and C3H10T1/2 as control cell lines.

2.3.5 Adhesion Blockade

To test the hypothesis that E-Selectin, ICAM-1, and VCAM-1 had a role in the increased adhesion, heterotypic cell adhesion assays were repeated as described above with one modification. HbmE cell monolayers were incubated for 1 hr with anti-E-selectin (12.5 µg/mL), anti-ICAM-1 (5 µg/mL), or anti-VCAM-1 (30 µg/mL) function blocking monoclonal antibodies (R&D Systems, Minneapolis, MN) and then washed twice with PBS prior to adding PCa cells to hbmE cell monolayers. Control wells were incubated for 1 hr with equivalent amounts of RPMI media. The concentrations of the antibodies employed were empirically determined over a range of concentrations.

2.3.6 Subcellular Fractionation

To determine if E-Selectin, ICAM-1, and/or VCAM-1 increased cell surface expression in response to calcium, cellular membranes were isolated from hbmE cells at 0, 1.5 and 3 hrs after calcium treatment. Briefly, cells were removed from tissue culture flasks with 5 mM EDTA then lysed with subcellular fractionation buffer [250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 7X...
Protease Inhibitor III cocktail (Roche Diagnostics, Indianapolis, IN)]. Cell lysates were then passed through a 25 Gauge needle and subjected to a series of centrifugations: 2 at 720 G for 10 min each, 10,000 G for 10 min, 100,000 G in an ultracentrifuge for 1 hr, then 100,000 G in an ultracentrifuge for 45 min. After each centrifugation, pellets were retained, re-suspended in subcellular fractionation buffer, and passed through a 25 G needle.

To quantify total protein, a BCA Protein Assay (Thermo Scientific, Logan, UT) was conducted and yielded an average of 0.6 µg/µl of protein from approximately 10 million cells. Western Blots were performed as described below and membranes were probed with antibodies specific for E-Selectin (1:500), VCAM-1 (1:600), and ICAM-1(1:500) mouse anti-goat polyclonal antibodies (R&D Systems, Minneapolis, MN). Goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (HRP) were used for secondary antibody incubations (Jackson Immunoresearch, West Grove, PA).

2.3.7 Western blot Analysis

To identify calcium responsive proteins in PCa cell lines, Western blot analysis was performed. Cells seeded in 48 well plates and cultured to 80% confluence were treated with 2.50 mM CaCl₂ for 3 or 24 hrs and then treated with lysis buffer containing: 10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 0.2% Triton X-100 and 7X protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were centrifuged at 10,000 G at 4°C for 7 min, 40 µg of total protein was loaded into each well of a 7.5% (w/v) sodium dodecyl sulfate polyacrylamide gel (SDS-Page), electrophoresed, and transferred to a nitrocellulose membrane by semi-dry Western blotting (Bio-Rad laboratories, Redmond, WA). The membrane was blocked in 5% (w/v) BSA in 0.05% (v/v) PBS-Tween (PBST) for 45 min then probed overnight using polyclonal antibodies specific for TRPV6 (1:500, Alomone,
Jerusalem, Israel), CaSR (1:500, Abcam, Cambridge, MA), Phosphoinositide 3-kinase (1:500, PI3K, Cell Signaling, Danvers, MA), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10000, Genscript, Piscataway, NJ). Goat anti-rabbit (Bio-Rad laboratories, Redmond, WA) and NeutraAvidin (Thermo Scientific, Rockville, IL) secondary antibodies conjugated to HRP were used, respectively. The bands on the membrane were visualized using enhanced chemiluminescence (Pierce Biotechnologies). Densitometric analysis was performed using a Bio-Rad acquisition system and data are expressed as mean integrated optical density (IOD) relative to GAPDH.

2.3.8 Migration Assay

To determine if calcium could enhance the migration of osteoblastic PCa cells, transwell migration assays were implemented using calcium as a chemoattractant. Calcium and serum starved (~12 hrs) C4-2 and C4-2B4 cells were detached from culture surfaces with 5 mM EDTA, re-suspended in calcium free DMEM, and loaded with 25 µM of Calcein-AM for 20 min. Subsequently, cells were washed twice with calcium free DMEM and 150,000 labeled cells were seeded on the top well of a tissue culture insert (8 micron pore, BD Falcon, San Jose, CA) in serum and calcium free DMEM. The bottom of the well contained either 10% (v/v) FBS (positive control) or 2.50 mM CaCl₂ as chemoattractants. After 24 hrs, tissue culture inserts were placed in 0.05% (v/v) trypsin/EDTA for 20 min to remove migrated cells from the bottom of the insert. Migrated cells were quantified by measuring fluorescence (485/515 nm). The sensitivity of this assay was predetermined by creating a standard curve employing known numbers of Calcein-AM labeled cells. In similar experiments, C3H10T1/2 fibroblasts were employed to access cell specificity.
2.3.9 Cell Invasion Assay

Invasion assays were employed to test the hypothesis that calcium enhances osteoblastic PCa cell invasion of hbmE cell monolayers. Invasion assays were performed similarly to migration assays but prior to seeding C4-2 cells, 50,000 hbmE cells were seeded in the top well of the tissue culture insert to create confluent monolayers. Calcium and serum starved (~12 hrs) C4-2 and C4-2B4 cells were detached from culture surfaces with 5 mM EDTA, re-suspended in calcium free DMEM, and loaded with 25 µM of Calcein-AM for 20 min. Subsequently, cells were washed twice with calcium free DMEM and 150,000 labeled cells were seeded on the top of the hbmE cell monolayers in serum and calcium free DMEM. The bottom of the well contained 10% (v/v) FBS, positive control, or 2.50 mM CaCl₂ as a chemoattractant. After 24 hrs, tissue culture inserts were placed in 0.05% (v/v) trypsin/EDTA for 20 min to remove invaded cells from the bottom of the insert. Cells removed from the bottom of the insert were quantified by measuring fluorescence (485/515 nm).

2.3.10 Wound Healing Assay

Wound healing assays were also employed to test the hypothesis that calcium enhances osteoblastic PCa cell migration. C4-2 cells were seeded in a 6 well tissue culture dish and allowed to become confluent. A P200 pipette tip was used to scratch down the center of the dish and medium was immediately removed and replaced with serum free T-Medium +/- 2.50 mM CaCl₂ or 5% (v/v) FBS, as a positive control; each condition was carried out in triplicate. Wounds were imaged at 0 and 24 hrs after the scratch was made. Mean width of the scratch was quantified using Image J by taking a minimum of 4 measurements along the length of the scratch then averaging the measurements across 3
separate images of the scratch. Data are expressed as wound width calculated from subtracting mean width at 24 hrs from mean width at 0 hrs.

2.3.11 RNA Extraction and Quantitative PCR

Trizol reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA from C4-2 and C4-2B4 monolayers 24 hrs after a 3 hr calcium exposure [2.50 mM CaCl$_2$]. RNA (500 ng) was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). RNA was denatured at 42°C for 50 min, and 200 U of SuperScript II reverse transcriptase enzyme was used in a final reaction volume of 20 µl. One micro-liter of cDNA was used for qPCR using SYBR Green (Applied Biosystems, Foster City, CA) and primers specific for TRPV6 and 18S (Table 2.1). SYBR Green and primers alone were used as a negative control. Sequences were created using the National Center for Biotechnology Information’s basic local alignment search tool. Sequences were then validated by qualitative PCR. Quantitative PCR was carried out using the RotorGene Real-Time DNA amplification system (Corbett Research, Sydney, Australia). Gene expression was normalized to 18S.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV6</td>
<td>ATGGTGATGCGGCTCATCAGTG</td>
<td>GTAGAAGTGGGCTAGCTCCTCG</td>
</tr>
<tr>
<td>18S</td>
<td>GAGAAACGGCTACC</td>
<td>CACCAGACTTGCCC</td>
</tr>
</tbody>
</table>

Table 2.1 Forward and Reverse primer sequences for Real Time PCR.

2.3.12 Indirect Immunocytochemistry

Cells were grown on glass coverslips or in 96 well dishes, washed with PBS and fixed with 2% (v/v) paraformaldehyde for 15 min in PBS. The cells were washed again with PBS, nonspecific staining was blocked with 3% (w/v) bovine serum albumin (BSA) for 30 min, and the cells were subjected to the conventional immunostaining procedure. Goat anti-rabbit
IgG conjugated to AlexaFluor 594 (1:1100, Molecular Probes, Carlsbad, CA) was used as a secondary antibody for TRPV6 staining. Anti-Mouse FITC (1:200, Santa Cruz, Santa Cruz, CA) was used as a secondary antibody for E-Selectin, ICAM-1, and VCAM-1 staining. Images were captured using an Olympus Fluo View (FV1000) confocal microscope with brightness set at 670 high voltage (HV) and 7% laser power for TRPV6 and 785 HV and 12% laser power for adhesion molecules. Cells incubated with secondary antibodies alone were used as a specificity control for all antibodies.

2.3.13 Statistical Analysis

All experiments were conducted in at least triplicate and repeated at least three times. Graphpad PRISM version 5.0 (GraphPad Software, San Diego CA) was used for statistical analysis. One-way analysis of variance (ANOVA) with a Bonferroni post hoc test was performed with experiments of multiple groups to compare all columns of data. Two-way ANOVA was used to analyze statistical significance of proliferation assays. Two tailed t-tests were used where appropriate. All data are expressed as mean ± standard deviation (SD) and were considered significant if p ≤ 0.05.

2.4 Results

2.4.1 The Effect of Elevated Extracellular Calcium on Osteoblastic PCa cell Proliferation

To test the hypothesis that increases in extracellular calcium enhance proliferation of PCa cells (C4-2 and C4-2B4 cell lines), two methods were employed. The first method, continuous calcium exposure wherein calcium was added to the culture system and not removed, and the second, transient calcium exposure, 3hrs/day. Proliferation was measured using CyQuant dye to measure the fluorescence of intact DNA. A standard curve was developed to determine mean fluorescence relative to cell number (Figure 2.1). Mean
fluorescence was thresholded at 256,000 cells which was equivalent to a mean fluorescence of 1689 nm. To determine the concentration of CaCl₂ to use in subsequent experiments a range of concentrations were tested between 1.25-2.50 mM. Cellular proliferation was significantly decreased compared to untreated control at all doses tested (Figure 2.2). The mean fluorescence of untreated control cells was outside the range of the standard curve after 48 hrs; however, even if the cells did not continue to proliferate, the mean fluorescence would still be significantly higher than cells treated with CaCl₂. Continuous elevations in CaCl₂, but not transient ((Figures 2.7 and 2.9), were found to significantly decrease C4-2 and C4-2B4 proliferation (Figures 2.3 and 2.4). These results were confirmed in parallel plates employing cell counts with Trypan blue exclusion and proliferation was decreased only at 96 hrs (Figure 2.5). C3H10T1/2 fibroblast proliferation was not altered when treated with continuous elevations in CaCl₂ suggesting this decrease in proliferation in response to elevations in CaCl₂ may be specific to PCa epithelial cells (Figure 2.6). In contrast, transient elevations in CaCl₂ did not alter C4-2 or C4-2B4 proliferation.
Figure 2.1 Cellular Proliferation Standard Curve: cell number relative to mean fluorescence. Cell numbers ranged from 0-256,000 and were quantified to develop a standard curve and identify the sensitivity of the CyQuant assay. Each closed circle represents the mean +/- SD of triplicate observations.
Figure 2.2 Continuous exposure to elevated CaCl$_2$ decreased C4-2 proliferation. C4-2 cells were seeded at 50% confluence in 96 well plates and carried in medium containing 1.25, 1.50, 1.75, or 2.50 mM CaCl$_2$. Every 24 hrs, cell number was quantified with CyQuant dye by measuring mean fluorescence (485/530 nm). Closed circles represent the proliferation of cells carried under normal growth conditions [1.25 mM CaCl$_2$]. Each point represents the mean +/- SD of triplicate observations. * indicates significantly different from all conditions, $p \leq 0.05$. 
Figure 2.3 Continuous exposure to elevated CaCl$_2$ decreased C4-2 proliferation. C4-2 cells were seeded at 50% confluence in 96 well dishes and carried in medium containing 2.50 mM CaCl$_2$. Every 48 hrs, cell number was quantified by measuring CyQuant Dye mean fluorescence (485/530 nm). Each point reflects the mean fluorescence +/- SD of triplicate observations for controls (closed circles) and cells treated with 2.50 mM CaCl$_2$ (closed squares). * indicates significantly different from control, p≤0.05, n=4
Continuous exposure to elevated CaCl$_2$ decreased C4-2B4 proliferation.

C4-2B4 cells were seeded at 50% confluence in 96 well dishes and exposed to 2.50 mM CaCl$_2$. Every 48 hrs, cell number was quantified with CyQuant Dye by measuring mean fluorescence (485/530 nm). Each point reflects the mean fluorescence +/- SD of triplicate observations for controls (closed circles) and cells treated with 2.50 mM CaCl$_2$ (closed squares). * indicates significantly different from control, p≤0.05.
Figure 2.5 Continuous exposure to elevated CaCl$_2$ decreased C4-2 proliferation. C4-2 cells were seeded at 50% confluence in 6 well dishes and carried in medium containing 2.50 mM CaCl$_2$. Every 24 hrs, cells were released from culture surfaces using trypsin and viable cells were quantified with a hemocytometer using trypan blue exclusion. Each point reflects the mean fluorescence +/- SD of triplicate observations for controls (closed circles) and cells treated with 2.50 mM CaCl$_2$ (closed squares). * indicates significantly different from control, p≤0.05.
Figure 2.6 Continuous exposure to elevated CaCl$_2$ did not alter fibroblast proliferation.

C3H10T1/2 fibroblasts were seeded at 50% confluence in 96 well dishes and carried in medium containing 2.50 mM CaCl$_2$. Every 24 hrs, cell number was quantified with CyQuant Dye by measuring mean fluorescence (485/530 nm). Each point reflects the mean fluorescence +/- SD of triplicate observations for controls (closed circles) and cells treated with 2.50 mM CaCl$_2$ (closed squares).
Figure 2.7 Transient exposure to elevated CaCl₂ did not alter C4-2 proliferation. C4-2 cells were seeded at 50% confluence in 96 well dishes and exposed to 2.50 mM CaCl₂ for 3 hrs/day. Every 48 hrs, cell number was quantified with CyQuant Dye by measuring mean fluorescence (485/530 nm). Each point reflects the mean fluorescence +/- SD of triplicate observations for controls (closed circles) and cells treated with 2.50 mM CaCl₂ (closed squares).
Figure 2.8 Transient exposure to elevated CaCl$_2$ did not alter C4-2B4 proliferation.

C4-2B4 cells were seeded at 50% confluence in 96 well dishes and exposed to 2.50 mM CaCl$_2$ for 3 hrs/day. Every 24 hrs, cell number was quantified with CyQuant Dye by measuring mean fluorescence (485/530 nm). Each point reflects the mean fluorescence +/- SD of triplicate observations for controls (closed circles) and cells treated with 2.50 mM CaCl$_2$ (closed squares).
2.4.2 The Effect of Elevated Extracellular Calcium on Cellular Activity

Since continuous elevations in CaCl₂ decreased the proliferation of PCa cell lines, *in vitro*, we tested the hypothesis that increased extracellular calcium enhances PCa cell survival. Cell survival was first challenged by carrying cells for 72 hrs in serum free medium, which decreased C4-2 cellular activity by 33% (Figure 2.9). Under these conditions, continuous or transient elevations in CaCl₂ [2.50 mM] did not alter cellular activity (Figure 2.10).

Cell survival was then challenged by culturing cells in the presence of excess ATP for 48 hrs. High concentrations of ATP are known to induce cell death [147]. After carrying the cells exposed to a broad range of ATP [0-3000 mM] it was determined that 3,000 mM significantly decreased cellular activity (47%), relative to untreated controls (Figure 2.11). Exposure to CaCl₂ [2.50 mM] did not enhance cellular activity in the presence of 3,000 mM of ATP (Figure 2.12) suggesting CaCl₂ did not enhance C4-2 cell survival.
Figure 2.9 Serum starvation decreased C4-2 cellular activity. C4-2 cells (8,000) were seeded in a 96 well plate. The next day, serum was reduced from 5% to either 1 or 0% serum. After 72 hrs under reduced serum conditions, cellular activity was quantified by measuring MTT reduction. The bars represent the mean optical density (OD) +/- SD of triplicate observations for cells carried in 1% serum conditions (white) and 0% serum conditions (checkered). * indicates significantly different from control, p≤0.05.
Figure 2.10 Extracellular calcium did not enhance the cellular activity of serum starved cells. Serum starved C4-2 cells were exposed to transient (t2.50) or continuous (c2.50) elevations in CaCl₂ (2.50 mM) for 72 hrs. Cellular activity was then quantified by measuring MTT reduction. The bars represent the mean OD +/- SD of triplicate observations for cells carried in 0% serum conditions (checkered), cells exposed to CaCl₂ [2.50 mM] for 3 hrs/day (gray), or cells continuously exposed to CaCl₂ [2.50 mM] (black).
Figure 2.11 ATP decreased C4-2 cellular activity. C4-2 cells were seeded in 96 well dishes and treated with a range of concentrations of ATP [3-3,000 mM]. After 48 hrs, medium was removed and cellular activity was quantified by measuring MTT reduction. The bars represent the mean OD +/- the SD of triplicate observations for each concentration of ATP used. * indicates significantly different from control, p≤0.05.
Figure 2.12 Elevations in extracellular calcium did not enhance the cellular activity of cells exposed to ATP. C4-2 cells were seeded in 96 well dishes and exposed to 3,000 mM ATP +/- 2.50 mM CaCl$_2$. After 48 hrs medium was removed and cellular activity was quantified by measuring MTT reduction. The bars represent the mean OD +/- the SD of triplicate observations of cells treated with 3,000 mM ATP (white) and cells treated with 3,000 mM ATP and exposed to elevations in extracellular calcium [2.50 mM] (black).
2.4.3 The Role of Extracellular Calcium in Osteoblastic PCa cell Adhesion

To test the hypothesis that elevations in extracellular calcium enhance PCa cell adhesion to hbmE cells heterotypic cell adhesion assays were employed. Observations from these studies suggested that heterotypic cell adhesion is decreased in the presence of elevated extracellular calcium [1.25 mM] (Figure 2.13). Pretreating either hbmE or PCa cells with 1.25 mM CaCl$_2$ prior to conducting adhesion assays yields disparate results. We observed a 77% increase in C4-2 to hbmE cell adhesion when hbmE cells were pre-exposed to elevated CaCl$_2$ [1.25 mM]. In contrast, adhesion was not altered when C4-2 cells were pre-exposed to elevated CaCl$_2$ (Figure 2.14). Adhesion of C4-2B4 cells to hbmE cells was also enhanced (59%) when hbmE cells were pre-exposed to elevated CaCl$_2$ but not when C4-2B4 cells were pre-exposed to CaCl$_2$ (Figure 2.15). To determine if these observations were specific to PCa cells, adhesion assays were repeated with HFOB and C3H10T1/2 cells. Adhesion of HFOB cells to hbmE cell monolayers was not altered when either cell line was pre-exposed to elevated CaCl$_2$ (Figure 2.16), however, C3H10T1/2 adhesion to hbmE cell monolayers was decreased when hbmE cells were pre-exposed to elevated CaCl$_2$ (Figure 2.17). Interestingly, when HFOB cells were used in place of hbmE cells, C4-2 cell adhesion was enhanced to HFOB cells by 22% (Figure 2.18).

To identify cell surface molecules responsible for increased adhesion, membrane fractions were isolated from hbmE cells treated with 1.25 mM of CaCl$_2$ 0, 1.5, and 3 hrs after treatment and analyzed by Western blot. This approach suggests that cell surface abundance of E-Selectin, VCAM-1, and ICAM-1 protein was increased 3, 4 and 100 fold, respectively (Figures 2.19-2.21). Parallel plates analyzed by immunocytochemistry, employing antibodies specific for E-Selectin, VCAM-1, and ICAM-1, also suggested CaCl$_2$ exposure
[1.25 mM] increased the cell surface presence of E-Selectin, VCAM-1, and ICAM-1 3 hrs after treatment (Figure 2.19-2.21).

To identify a role for E-Selectin, VCAM-1, and ICAM-1 in heterotypic cell adhesion following CaCl₂ exposure, hbmE cells were incubated with function blocking antibodies prior to the start of adhesion assays. Antibody concentrations were empirically determined over a range of concentrations [0-30 µg/ml]. It was determined that 12.5 µg/ml of E-selectin, 30 µg/ml of VCAM-1, and 5 µg/ml of ICAM-1 antibodies were sufficient to block at least 56% adhesion between C4-2 and hbmE cells. (Figures 2.22-2.24). An isotype specific antibody was employed, as a control for antibody specificity, which did not alter adhesion (Figure 2.25). When hbmE cells were pre-treated with CaCl₂ then incubated with 12.5 µg/ml of E-Selectin antibody prior to the addition of C4-2 cells, heterotypic cell adhesion was decreased 44% (Figure 2.26). Similarly, incubation with VCAM-1 antibodies decreased C4-2 cell adhesion 49% (Figure 2.27) while ICAM-1 antibodies suppressed adhesion by 45% (Figure 2.28). Incubating hbmE cells with various combinations of E-Selectin, ICAM-1, and VCAM-1 did not further decrease heterotypic cell adhesion (Figure 2.29).
Figure 2.13 Elevated extracellular calcium decreased C4-2 cell adhesion to hbmE cell monolayers. Calcein-AM labeled C4-2 cells were added to hbmE cell monolayers. Cultures were subsequently cultured in the presence of CaCl$_2$ [2.50 mM] for 3 hrs. Wells were washed 3X with PBS, gently agitated to remove un-adherent cells, and lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations for untreated cells (white) and cells exposed to 2.50 mM CaCl$_2$ (black). * indicates significantly different from control, p≤0.05.
Figure 2.14 Calcium exposure to hbmE cell monolayers enhanced C4-2 cell adhesion.

HbmE or C4-2 cells were exposed to 1.25 mM CaCl$_2$ for 3 hrs then C4-2 cells were loaded with Calcein-AM and added to hbmE cell monolayers. After 3 hrs, wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations for adherent C4-2 cells added to untreated hbmE cell monolayers (white), CaCl$_2$ treated hbmE cell monolayers (green), or adherent C4-2 cells pre-exposed to CaCl$_2$ then added to untreated hbmE cell monolayers (gray). The +/- indicates pretreatment with or without CaCl$_2$, * indicates significantly different from control, p≤0.05.
Figure 2.15 Calcium exposure to hbmE cell monolayers enhanced C4-2B4 cell adhesion.

HbmE or C4-2B4 cells were exposed to 1.25 mM CaCl\textsubscript{2} for 3 hrs then C4-2 cells were loaded with Calcein-AM and added to hbmE cell monolayers. After 3 hrs, wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2B4 cells added to untreated hbmE cell monolayers (white), CaCl\textsubscript{2} treated hbmE cell monolayers (green), or adherent C4-2B4 cells pre-exposed to CaCl\textsubscript{2} then added to untreated hbmE cell monolayers (black). The +/- indicates pretreatment with or without CaCl\textsubscript{2}, * indicates significantly different from control, p≤0.05.
Figure 2.16 Calcium exposure to hbmE cell monolayers did not enhance HFOB cell adhesion. HbmE or HFOB cells were exposed to 1.25 mM CaCl₂ for 3 hrs then HFOB cells were loaded with Calcein-AM and added to hbmE cell monolayers. After 3 hrs, wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent HFOB cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent HFOB cells added to untreated hbmE cell monolayers (white), CaCl₂ treated hbmE cell monolayers (green), or adherent HFOB cells pre-exposed to CaCl₂ then added to untreated hbmE cell monolayers (dark gray). The +/- indicates pretreatment with or without CaCl₂, * indicates significantly different from control, p≤0.05.
Figure 2.17 Calcium exposure to hbmE cell monolayers decreased fibroblast cell adhesion. HbmE or C3H10T1/2 cells were exposed to 1.25 mM CaCl$_2$ for 3 hrs then C3H10T1/2 cells were loaded with Calcein-AM and added to hbmE cell monolayers. After 3 hrs, wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C3H10T1/2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C3H10T1/2 cells added to untreated hbmE cell monolayers (white), CaCl$_2$ treated hbmE cell monolayers (green), or adherent C3H10T1/2 cells pre-exposed to CaCl$_2$ and added to untreated hbmE cell monolayers (light gray). The +/- indicates pretreatment with or without CaCl$_2$, * indicates significantly different from control, p≤0.05.
Figure 2.18 Calcium exposure to C4-2 cells enhanced their adhesion to HFOB cell monolayers. HFOB or C4-2 cells were exposed to 1.25 mM CaCl₂ for 3 hrs then C4-2 cells were loaded with Calcein-AM and added to HFOB cell monolayers. After 3 hrs, wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to untreated HFOB cell monolayers (white), CaCl₂ treated HFOB cell monolayers (green), or adherent C4-2 cells pre-exposed to CaCl₂ and added to untreated HFOB cell monolayers (gray). The +/- indicates pretreatment with or without CaCl₂, * indicates significantly different from control, p≤0.05.
A

**115 kDa**

![Graph showing Mean IOD](image)

- **Control**
- **0 Hrs.**
- **1.5 Hrs.**
- **3 Hrs.**

B

**Control**

**3 hrs**

**Neg. Control**

![Images of fluorescent analysis](image)
Figure 2.19 Extracellular calcium enhanced E-Selectin cell surface abundance on hbmE cells. (A) Representative Western blot image and summary bar graph illustrating E-Selectin protein expression in membrane fractions of hbmE cells treated with CaCl₂. Membrane fractions were isolated from hbmE cells at 0, 1.5 and 3 hrs after a 3 hr exposure to CaCl₂ [1.25 mM]. Western blot analysis was conducted and membranes were probed with antibodies specific to E-Selectin. The bars represent the mean IOD +/- SD of triplicate observations of E-Selectin protein expression in untreated hbmE cells (white), hbmE cells 0 hrs after CaCl₂ exposure (light gray), hbmE cells 1.5 hrs after CaCl₂ exposure (gray), and hbmE cells 3 hrs after CaCl₂ exposure (green). * indicates significantly different from untreated control, p≤0.05, n=3 (B) Representative confocal micrographs depicting E-Selectin cell surface localization on untreated hbmE cells (control) and hbmE cells 3 hrs after a 3 hr exposure to CaCl₂ [1.25 mM]. HbmE cells were grown on glass coverslips and fixed with 2% paraformaldehyde, non-specific staining was blocked with 3% (w/v) BSA and cells were subjected to the conventional indirect immunostaining procedures using primary antibodies specific to E-Selectin. Anti- Mouse FITC was used as secondary antibody (Green). Neg. Control image illustrates cells incubated with secondary antibody only. Images were captured using an Olympus Fluo View (FV1000) confocal microscope with a 40X objective. Green indicates E-Selectin, Blue indicates DAPI nuclear stain, scale bar= 50 µm
Figure 2.20 Extracellular calcium enhanced VCAM-1 cell surface abundance on hbmE cells. (A) Representative Western blot image and summary bar graph illustrating VCAM-1 protein expression in membrane fractions of hbmE cells treated with CaCl$_2$. Membrane fractions were isolated from hbmE cells at 0, 1.5 and 3 hrs after a 3 hr exposure to CaCl$_2$ [1.25 mM]. Western blot analysis was conducted and membranes were probed with antibodies specific to VCAM-1. The bars represent the mean IOD +/- SD of triplicate observations of VCAM-1 protein expression in untreated hbmE cells (white), hbmE cells 0 hrs after CaCl$_2$ exposure (light gray), hbmE cells 1.5 hrs after CaCl$_2$ exposure (gray), and hbmE cells 3 hrs after CaCl$_2$ exposure (green). * indicates significantly different from untreated control, p≤0.05, n=3 (B) Representative confocal micrographs depicting VCAM-1 cell surface localization on untreated hbmE cells (control) and hbmE cells 3 hrs after a 3 hr exposure to CaCl$_2$ [1.25 mM]. HbmE cells were grown on glass coverslips and fixed with 2% paraformaldehyde, non-specific staining was blocked with 3% (w/v) BSA and cells were subjected to the conventional indirect immunostaining procedures using primary antibodies specific to VCAM-1. Anti- Mouse FITC was used as secondary antibody (Green). Images were captured using an Olympus Fluo View (FV1000) confocal microscope with a 40X objective. Green indicates VCAM-1, Blue indicates DAPI nuclear stain, scale bar= 50 µm.
Figure 2.21 Extracellular calcium enhanced ICAM-1 cell surface abundance on hbmE cells.  (A) Representative Western blot image and summary bar graph illustrating ICAM-1 protein expression in membrane fractions of hbmE cells treated with CaCl$_2$. Membrane fractions were isolated from hbmE cells at 0, 1.5 and 3 hrs after a 3 hr exposure to CaCl$_2$ [1.25 mM]. Western blot analysis was conducted and membranes were probed with antibodies specific to ICAM-1. The bars represent the mean IOD +/- SD of triplicate observations of ICAM-1 protein expression in untreated hbmE cells (white), hbmE cells 0 hrs after CaCl$_2$ exposure (light gray), hbmE cells 1.5 hrs after CaCl$_2$ exposure (gray), and hbmE cells 3 hrs after CaCl$_2$ exposure (green). * indicates significantly different from untreated control, p≤0.05, n=3 (B) Representative confocal micrographs depicting ICAM-1 cell surface localization on untreated hbmE cells (control) and hbmE cells 3 hrs after a 3 hr exposure to CaCl$_2$ [1.25 mM]. HbmE cells were grown on glass coverslips and fixed with 2% paraformaldehyde, non-specific staining was blocked with 3% (w/v) BSA and cells were subjected to the conventional indirect immunostaining procedures using primary antibodies specific to ICAM-1. Anti- Mouse FITC was used as secondary antibody (Green). Images were captured using an Olympus Fluo View (FV1000) confocal microscope with a 40X objective. Green indicates ICAM-1, Blue indicates DAPI nuclear stain, scale bar= 50 µm
Figure 2.22 E-Selectin antibodies decreased C4-2 cell adhesion to hbmE cell monolayers. HbmE cell monolayers were incubated with 0, 12.5, 25, or 50 µg/ml anti-E-Selectin function blocking antibodies for 1 hr then overlaid with C4-2 cells loaded with Calcein-AM. After 3 hrs, the wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to untreated hbmE cell monolayers (white) or added to hbmE cell monolayers incubated with 12.5 (checkered), 25 (diagonal stripes), or 50 (vertical stripes) µg/ml anti-E-Selectin function blocking antibodies.

* indicates significantly different from untreated control, p≤0.05.
Figure 2.23 VCAM-1 antibodies decreased C4-2 cell adhesion to hbmE cell monolayers.

HbmE cell monolayers were incubated with 0, 15, or 30 µg/ml anti-VCAM-1 function blocking antibodies for 1 hr then overlaid with C4-2 cells loaded with Calcein-AM. After 3 hrs, the wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to untreated hbmE cell monolayers (white) or added to hbmE cell monolayers incubated with 15 (checked) or 30 (stripes) µg/ml anti-VCAM-1 function blocking antibodies. * indicates significantly different from untreated control, $p\leq 0.05$. 
Figure 2.24 ICAM-1 antibodies decreased C4-2 cell adhesion to hbmE cell monolayers.

HbmE cell monolayers were incubated with 0, 5, or 10 µg/ml anti-ICAM-1 function blocking antibodies for 1 hr then overlaid with C4-2 cells loaded with Calcein-AM. After 3 hrs, the wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to untreated hbmE cell monolayers (white) or added to hbmE cell monolayers incubated with 5 (checkered) or 10 (stripes) µg/ml anti-ICAM-1 function blocking antibodies. * indicates significantly different from untreated control, p≤0.05.
Figure 2.25 Non-specific isotype control antibodies did not effect C4-2 cell adhesion to hbmE cell monolayers. HbmE cells were incubated with 0, 5, 12.5, or 30 µg/ml of anti-mouse IgG1 antibodies for 1 hr then overlaid with C4-2 cells loaded with Calcein-AM. After 3 hrs, the wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to untreated hbmE cell monolayers (white) or adherent C4-2 cells added to hbmE cell monolayers incubated with 5 (checkered), 12.5 (vertical stripes), or 30 (horizontal stripes) µg/ml anti-mouse IgG1 antibodies.
Figure 2.26 E-Selectin function blocking antibodies disrupted C4-2 cell adhesion to hbmE cell monolayers. HbmE cell monolayers exposed to elevated CaCl$_2$ [1.25 mM] for 3 hrs were incubated with 0 or 12.5 µg/ml anti-E-Selectin function blocking antibodies for 1 hr, then overlaid with C4-2 cells loaded with Calcein-AM. After 3 hrs, wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to untreated hbmE cell monolayers (white), CaCl$_2$ treated hbmE cell monolayers (green), hbmE cell monolayers incubated with 12.5 µg/ml anti-E-Selectin function blocking antibodies (black), and CaCl$_2$ treated hbmE cell monolayers incubated with 12.5 µg/ml anti-E-Selectin function blocking antibodies (checkered green). The +/- indicates pretreatment with or without CaCl$_2$ and E-Selectin antibody. * indicates significantly different from untreated control, p≤0.05.
Figure 2.27 VCAM-1 function blocking antibodies disrupted C4-2 cell adhesion to hbmE cell monolayers. HbmE cell monolayers exposed to elevated CaCl₂ [1.25 mM] for 3 hrs were incubated with 0 or 30 µg/ml anti-VCAM-1 function blocking antibodies for 1 hr, then overlaid with C4-2 cells loaded with Calcein-AM. After 3 hrs, the wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to untreated hbmE cell monolayers (white), CaCl₂ treated hbmE cell monolayers (green), hbmE cell monolayers incubated with 30 µg/ml anti-VCAM-1 function blocking antibodies (black), and CaCl₂ treated hbmE cell monolayers incubated with 30 µg/ml anti-VCAM-1 function blocking antibodies (green checkered). The +/- indicates pretreatment with or without CaCl₂ and VCAM-1 antibody. * indicates significantly different from untreated control, p≤0.05.
ICAM-1 function blocking antibodies disrupted C4-2 cell adhesion to hbmE cell monolayers. HbmE cell monolayers exposed to elevated CaCl₂ [1.25 mM] for 3 hrs were incubated with 0 or 5 µg/ml anti-ICAM-1 function blocking antibodies for 1 hr, then overlaid with C4-2 cells loaded with Calcein-AM. After 3 hrs, the wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to untreated hbmE cell monolayers (white), CaCl₂ treated hbmE cell monolayers (green), hbmE cell monolayers incubated with 5 µg/ml anti-ICAM-1 function blocking antibodies (black), and CaCl₂ treated hbmE cell monolayers incubated with 5 µg/ml anti-ICAM-1 function blocking antibodies (checkered green). The +/- indicates pretreatment with or without CaCl₂ and ICAM-1 antibody. * indicates significantly different from untreated control, p≤0.05.
Mixtures of function blocking antibodies did not further disrupt C4-2 cell adhesion to hbmE cell monolayers. HbmE cell monolayers were incubated for 1 hr with function blocking antibodies specific to E-Selectin, ICAM-1, and/or VCAM-1, indicated by +/-, and then overlaid with C4-2 cells loaded with Calcein-AM. After 3 hrs, wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% NP-40. Adherent C4-2 cells were quantified by measuring fluorescence (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of adherent C4-2 cells added to either untreated hbmE cell monolayers (white), hbmE cell monolayers incubated with 12.5 µg/ml anti-E-Selectin function blocking antibodies (dotted), hbmE cell monolayers incubated with 30 µg/ml anti-VCAM-1 function blocking antibodies (checkered), hbmE cell monolayers incubated with 5 µg/ml anti-ICAM-1 function blocking antibodies (diagonal stripes), hbmE cell monolayers incubated with 12.5 µg/ml anti-E-Selectin and 5 µg/ml anti-ICAM-1 function blocking antibodies (vertical stripes), or hbmE cell monolayers incubated with all three function blocking antibodies (black). * Indicates significantly different from all conditions, # Indicates significantly different from E-Selectin treatment, p≤0.05.
2.4.4 The Effect of Elevated Extracellular Calcium on Osteoblastic PCa cell Migration

Transwell migration assays were employed to test the hypothesis that increased extracellular calcium enhances osteoblastic PCa cell migration. To test this hypothesis calcein-AM labeled C4-2, C4-2B4, or C3H10T1/2 cells were placed on tissue culture inserts. Migration through the insert was induced by culturing the cells in the presence of CaCl₂ [2.50 mM] or 10% FBS (positive control, containing 0.05 mM CaCl₂), and compared to 0% FBS (negative control). The presence of CaCl₂ enhanced C4-2 cell migration by 52% compared to serum free conditions (Figure 2.30). CaCl₂ also enhanced C4-2B4 cell migration by 58%, which was comparable to that induced by 10% FBS (Figure 2.31). CaCl₂ did not alter C3H10T1/2 cellular migration (Figure 2.32).
Figure 2.30 C4-2 cell migration was enhanced by calcium. Calcium and serum starved (~12 hrs) C4-2 cells, loaded with 25 μM of Calcein-AM, were seeded on top of a tissue culture insert, in serum and calcium free DMEM. The insert was placed into a well containing either 10% (v/v) FBS or 2.5 mM CaCl₂ as a chemoattractant; control wells contained 0% (v/v) FBS. After 24 hrs, migrated cells were removed from the bottom of the insert with 0.05% (v/v) trypsin/EDTA and quantified by measuring fluorescence intensity (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of migrated C4-2 cells in response to serum free conditions (white), 10% (v/v) FBS (green), and 2.5 mM CaCl₂ (black) chemoattractants. * indicates significantly different from 0% and 10% FBS conditions, p≤0.05.
Figure 2.31 C4-2B4 cell migration was enhanced by calcium. Calcium and serum starved (~12 hrs) C4-2B4 cells, loaded with 25 µM of Calcein-AM, were seeded on top of a tissue culture insert, in serum and calcium free DMEM. The insert was placed into a well containing either 10% (v/v) FBS or 2.5 mM CaCl₂ as a chemoattractant; control wells contained 0% (v/v) FBS. After 24 hrs, migrated cells were removed from the bottom of the insert with 0.05% (v/v) trypsin/EDTA and quantified by measuring fluorescence intensity (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of migrated C4-2B4 cells in response to serum free conditions (white), 10% (v/v) FBS (green), and 2.5 mM CaCl₂ (black). * indicates significantly different from 0% FBS conditions, p≤0.05.
Figure 2.32 C3H10T1/2 cell migration was not enhanced by calcium. Calcium and serum starved (~12 hrs) C3H10T1/2 cells, loaded with 25 µM of Calcein-AM, were seeded on top of a tissue culture insert, in serum and calcium free DMEM. The insert was placed into a well containing either 10% (v/v) FBS or 2.5 mM CaCl₂ as a chemoattractant; control wells contained 0% (v/v) FBS. After 24 hrs, migrated cells were removed from the bottom of the insert with 0.05% (v/v) trypsin/EDTA and quantified by measuring fluorescence intensity (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of migrated C3H10T1/2 cells in response to serum free conditions (white), 10% (v/v) FBS (green), and 2.5 mM CaCl₂ (black).
2.4.5 The Effect of Elevated Extracellular Calcium on Wound Closure

A wound healing assay was employed to measure PCa cell motility and test the hypothesis that increased extracellular calcium enhances osteoblastic PCa cell migration. To test this hypothesis, a scratch, using a P200 pipette tip, was made down the center of wells containing PCa cell monolayers. After washing, the cells were carried in T-medium containing either 5% (v/v) or 0% (v/v) serum +/- 2.50 mM CaCl₂. Digital images were captured at 0 and 24 hrs after wounding. Wound width was calculated from subtracting mean width at 24 hrs from mean width at 0 hrs. Observations suggested that the mean width of the wound was decreased by the presence of increased CaCl₂ (continuous and transient) by 33% and 44%, respectively, compared to serum free conditions (Figure 2.33).
Figure 2.33 C4-2 cell wound closure was enhanced by CaCl₂. C4-2 cells were seeded in a 6 well tissue culture dish. When cells were confluent, a P200 pipette tip was used to scratch down the center of each dish. After wounding, dishes were rinsed and replaced with serum free T-Medium +/- 2.50 mM CaCl₂ or 5% (v/v) serum (positive control). Cultures were exposed to elevations in extracellular calcium continuous (24 hrs) or transient (3 hrs). The average width of each wound was quantified by taking four measurements along the length of each wound and averaging the measurements across three fields, spanning the entire length of the wound. Wound closure was calculated from mean width at 0 and 24 hrs. A) The bars represent triplicate observations of the mean width of wounds exposed to serum free conditions (white), 5% FBS (green), a 24 hr elevation in CaCl₂ (black), and a 3 hr elevation in CaCl₂ (gray). B) Representative images of cultures 24 hrs post-wounding. * indicates significantly different from all treatment groups, p≤0.05.
2.4.6 The Effect of Elevated Extracellular Calcium on Osteoblastic PCa cell Invasion

To test the hypothesis that elevated levels of extracellular calcium enhances osteoblastic PCa cell invasion of hbmE cell monolayers, transwell invasion assays were employed. The top of tissue culture inserts were prepared with monolayers of hbmE cells. PCa cells labeled with Calcein-AM were subsequently placed on top of the hbmE cell monolayers. Using CaCl$_2$ [2.50 mM] as a chemoattractant, we observed a 36% increase in C4-2 cell invasion of hbmE cell monolayers compared to calcium free conditions (Figure 2.34). Similar observations were made when C4-2B4 cells were employed (Figure 2.35).
Figure 2.34 C4-2 invasion of hbmE cell monolayers was enhanced by CaCl$_2$. HbmE cell monolayers covering the top of tissue culture inserts were overlaid with calcium and serum starved (~12 hrs) Calcein-AM labeled C4-2 cells. The bottom well contained either 0% (v/v), 10% (v/v) FBS, or 2.5 mM CaCl$_2$ as chemoattractants. After 24 hrs, C4-2 cells on the bottom of the insert were removed with 0.05% (v/v) trypsin/EDTA and quantified by measuring fluorescence intensity (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of C4-2 cells that moved through hbmE cell monolayers in response to serum free conditions (white), 10% (v/v) FBS (green), and 2.5 mM CaCl$_2$ (black). * indicates significantly different from 0% FBS conditions, p≤0.05.
Figure 2.35 C4-2B4 invasion of hbmE cell monolayers was enhanced by CaCl$_2$. HbmE cell monolayers covering the top of tissue culture inserts were overlaid with calcium and serum starved (~12 hrs) Calcein-AM labeled C4-2B4 cells. The bottom well contained either 0% (v/v), 10% (v/v) FBS, or 2.5 mM CaCl$_2$ as chemoattractants. After 24 hrs, C4-2B4 cells on the bottom of the insert were removed with 0.05% (v/v) trypsin/EDTA and quantified by measuring fluorescence intensity (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations of C4-2B4 cells that moved through hbmE cell monolayers in response to serum free conditions (white), 10% (v/v) FBS (green), and 2.5 mM CaCl$_2$ (black). * indicates significantly different from 0% FBS conditions, p≤0.05.
2.4.7 The Effect of Elevated Extracellular Calcium on TRPV6 mRNA and Protein Expression

To identify the effect of increased extracellular calcium on TRPV6 mRNA, protein expression, and localization osteoblastic PCa cells, C4-2 and C4-2B4 cells, were treated with CaCl$_2$ [2.50 mM] and analyzed by quantitative PCR (qPCR), Western blot, and indirect immunocytochemistry. QPCR suggested TRPV6 steady state mRNA expression was enhanced in C4-2 and C4-2B4 cell lines 79 and 76%, respectively (Figures 2.36 and 2.38). Western blot analysis suggested that TRPV6 protein expression was enhanced 2 fold in C4-2 cells (Figure 2.37). Immunocytochemistry suggested elevated CaCl$_2$ visually increased TRPV6 cell surface localization in C4-2 cells (Figure 2.37). In contrast, TRPV6 protein expression, via Western blot, in C4-2B4 cells was unchanged in response to elevated CaCl$_2$ and immunocytochemistry suggested there was no visual change in TRPV6 localization (Figure 2.38). In addition, protein expression of the CaSR and PI3K were also significantly enhanced in C4-2 cells (Figure 2.37). In comparison, only CaSR protein expression was enhanced in C4-2B4 cells (Figure 2.39). Protein expression returned to control levels 48 hrs post CaCl$_2$ exposure, except for the CaSR which remained elevated in both cell lines. No significant changes in protein expression were detected 72 hrs following exposure to CaCl$_2$ (data not shown). These experiments were repeated employing continuous exposure to CaCl$_2$, interestingly C4-2B4 cells had significant increases in TRPV6 protein expression at 48 hrs. In contrast, TRPV6 protein expression in C4-2 cells, was unchanged at 24 hrs and significantly decreased by 48 hrs. On the other hand, CaSR protein expression was significantly enhanced at 24 hrs but returned to basal levels by 48 hrs (data not shown).
Figure 2.36 TRPV6 expression was increased in C4-2 cells exposed to CaCl2. A) TRPV6 steady state mRNA expression. Total RNA was extracted from C4-2 cells 24 hrs after a 3 hr exposure to CaCl2 [2.50 mM]. Gene expression was standardized to 18S RNA and data is expressed relative to untreated cells. The bars represent TRPV6 steady state mRNA expression +/- SD of triplicate observations of untreated C4-2 cells (white) and C4-2 cells exposed to CaCl2 (black). B) Representative Western blot image and summary bar graph illustrating TRPV6 protein expression. Protein was isolated from C4-2 cells 24 hrs after a 3 hr exposure with CaCl2 [2.50 mM] and a Western blot analysis was conducted. Protein expression was normalized to GAPDH protein expression. The bars represent the mean IOD +/- SD of triplicate observations of TRPV6 protein expression relative to GAPDH in untreated C4-2 cells (white) and C4-2 cells exposed to CaCl2 (black). * indicates significantly different from untreated control, p≤0.05. C) Representative micrographs of TRPV6 expression on C4-2 cells 24 hrs after a 3hr exposure to CaCl2 [2.50 mM]. C4-2 cells were grown in 96 well dishes and treated with CaCl2 for 3 hrs. After 24hrs, the cells were fixed with 2% paraformaldehyde and incubated with antibodies specific for TRPV6. Goat anti-rabbit IgG conjugated to AlexaFluor 594 (1:1100) was used as a secondary antibody (Red staining). Negative control= cells incubated with secondary antibody only. Images were captured using an Olympus IX71 inverted microscope with a 20X objective using Spot Diagnostic software. Scale bar = 0.01 mm
Figure 2.37 CaSR and PI3K protein expression in C4-2 cells was enhanced in response to CaCl₂. Total protein was isolated from C4-2 cells 24 hrs after a 3 hr exposure to CaCl₂ [2.50 mM]. Membranes were probed with antibodies specific to CaSR and PI3K and normalized to GAPDH. A) Representative Western blot image and summary bar graph illustrating CaSR protein expression. The bars represent mean IOD +/- SD of triplicate observations of CaSR protein expression relative to GAPDH in untreated C4-2 cells (white) and C4-2 cells exposed to CaCl₂ (black). B) Representative Western blot image and summary bar graph illustrating PI3K protein expression. The bars represent mean IOD +/- SD of triplicate observations of PI3K protein expression relative to GAPDH in untreated C4-2 cells (white) and C4-2 cells exposed to CaCl₂ (black). * indicates significantly different from untreated control, p≤0.05.
Figure 2.38 TRPV6 mRNA expression was increased in C4-2B4 cells exposed to CaCl₂.

A) TRPV6 steady state mRNA expression. Total RNA was extracted from C4-2B4 cells 24 hrs after a 3 hr exposure to CaCl₂ [2.50 mM]. Gene expression was standardized to 18S RNA and data is expressed relative to untreated cells. The bars represent TRPV6 steady state mRNA expression +/- SD of triplicate observations of untreated C4-2B4 cells (white) and C4-2B4 cells exposed to CaCl₂ (black). B) Representative Western blot image and summary bar graph illustrating TRPV6 protein expression. Protein was isolated from C4-2B4 cells 24 hrs after a 3 hr exposure with CaCl₂ [2.50 mM] and a Western blot analysis was conducted. Protein expression was expressed relative to GAPDH. The bars represent the mean +/- SD of triplicate observations of TRPV6 protein expression relative to GAPDH in untreated C4-2B4 cells (white) and C4-2B4 cells exposed to CaCl₂ (black). * indicates significantly different from untreated control, p≤0.05. C) Representative micrographs of TRPV6 expression on C4-2B4 cells 24 hrs after a 3hr exposure to CaCl₂ [2.50 mM]. C4-2B4 cells were grown in 96 well dishes and treated with CaCl₂ for 3 hrs. After 24hrs, the cells were fixed with 2% paraformaldehyde and incubated with antibodies specific for TRPV6. Goat anti-rabbit IgG conjugated to AlexaFluor 594 (1:1100) was used as a secondary antibody (Red staining). Negative control= cells incubated with secondary antibody only. Images were captured using an Olympus IX71 inverted microscope with a 20X objective using Spot Diagnostic software. Scale bar = 0.01 mm
Figure 2.39 CaSR expression in C4-2B4 cells was enhanced in response to CaCl$_2$. Total protein was isolated from C4-2B4 cells 24 hrs after a 3 hr exposure to CaCl$_2$ [2.50 mM]. Membranes were probed with antibodies specific to CaSR and PI3K and normalized to GAPDH. A) Representative Western blot image and summary bar graph illustrating CaSR protein expression. The bars represent mean IOD +/- SD of triplicate observations of CaSR protein expression relative to GAPDH in untreated C4-2B4 cells (white) and C4-2B4 cells exposed to CaCl$_2$ (black). B) Representative Western blot image and summary bar graph illustrating PI3K protein expression. The bars represent mean IOD +/- SD of triplicate observations of PI3K protein expression relative to GAPDH in untreated C4-2B4 cells (white) and C4-2B4 cells exposed to CaCl$_2$ (black). * indicates significantly different from untreated control, p≤0.05.
2.5 Discussion

Extracellular calcium has been hypothesized to have role in cancer progression [52, 145, 146] although it is not known whether increases in extracellular calcium increase the metastatic potential of osteoblastic PCa cells. We aimed to identify the role of transient increases in extracellular calcium on adhesion, migration, and proliferation of osteoblastic PCa cells, *in vitro*. Our observations suggested that transient increases in extracellular calcium decreased proliferation and had no effect on PCa cell viability. Calcium exposure during adhesion assays did not alter PCa cell to hbmE cell adhesion, however, pretreatment of hbmE cells with extracellular calcium enhanced C4-2 and C4-2B4 PCa cell adhesion. We also observed that calcium, as a chemoattractant, enhanced osteoblastic PCa cell invasion of hbmE cell monolayers. TRPV6 expression was enhanced following transient elevations in extracellular calcium suggesting a possible cellular mechanism responsible for these changes in adhesion and invasion. This is the first study to demonstrate that transient increases in extracellular calcium enhance osteoblastic PCa cell adhesion and invasion.

Elevated levels of extracellular calcium have been reported to have both stimulatory and inhibitory actions on cellular proliferation depending on cell type and concentration of CaCl$_2$ [104]. Our observations suggested that increased extracellular calcium decreased proliferation of C4-2 and C4-2B4 PCa cell lines. Liao et al. concluded that high extracellular calcium [2.5 mM] significantly enhanced proliferation of PC-3 PCa cells compared to low extracellular calcium [0.5 mM] [52]. In contrast, we compared high levels of calcium [2.5 mM] to basal [1.25 mM] levels perhaps offering one explanation for the differences observed. Additionally, PC-3 cells have different characteristics such as an osteolytic nature compared to C4-2 cells which are osteoblastic in nature. This suggests PC-3 cells may handle
extracellular calcium differently than osteoblastic PCa cells. There is literature to suggest that high levels of extracellular calcium inhibit the proliferation of colon and parathyroid cells [104, 148-150] which is consistent with our observations. In one study, colonic epithelial cells isolated from patients with colon cancer, cultured in the presence of 2.2 mM CaCl$_2$, had a slower rate of proliferation than colon cells cultured in the presence of 0.1 mM CaCl$_2$[151]. Nonetheless, there have been no other studies suggesting that increases in extracellular calcium decrease the proliferation of osteoblastic PCa cell lines.

While proliferation was decreased in response to high calcium, another study has implicated a role for extracellular calcium providing an anti-apoptotic effect [152]. To explore this possibility we tested the hypothesis that increases in extracellular calcium enhance osteoblastic PCa cell viability. We employed MTT assays to assess cellular viability, this assay is limited by an overestimation of the number of viable cells [153]. However, increased extracellular calcium did not enhance cell viability when cells were challenged with serum starvation or high levels of ATP. Lin et al. demonstrated that elevated extracellular calcium, exceeding 7 mM, increased cell survival of AT-3 PCa cells challenged with ATP [152]. Although this concentration is 5.5 fold over normal physiological concentrations, in vivo experiments have demonstrated that extracellular calcium concentrations can reach levels of 30 mM suggesting AT-3 PCa cells can sustain these high levels. In contrast, when our osteoblastic PCa cells were cultured in extracellular calcium concentrations between 5-10 mM we observed cell death within 24 hrs. Therefore it is unlikely that an increase in extracellular calcium exceeding 2.50 mM would have produced a different outcome. One distinction between C4-2 cells and AT-3 cells are that AT-3 cells were derived from a malignant prostate tumor isolated from an adult Copenhagen rat [154],
while C4-2 cells are human PCa cell lines isolated from murine bone which may explain the differences in calcium handling between the two cell lines. These observations suggest that increases in extracellular calcium act on other cellular mechanisms responsive to calcium in osteoblastic PCa cells.

During bone turnover, calcium exiting or entering the bone environment will encounter sinusoids, comprised largely of bone marrow endothelial cells [131, 133]. We employed heterotypic cell adhesion assays to attempt to mimic this interaction in vitro. Our data suggested that osteoblastic PCa cell adhesion to hbmE cell monolayers was enhanced when hbmE cells were pretreated with calcium. Several studies have suggested a multitude of calcium responsive adhesion molecules, such as selectins, integrins, and cadherins, on endothelial cells are required for metastatic cancer cell adhesion [139, 155, 156]. PCa cells have previously been shown to preferentially adhere to hbmE cells compared to human umbilical vein endothelial cells (HUVEC) [134]. One possible explanation is that E-Selectin and VCAM-1 expression is barely detectable on HUVECs while basal levels of both adhesion molecules are readily detectable on hbmE cells, analyzed by flow cytometry [140]. Additionally, HUVECs are not found in the bone microenvironment which also may explain why PCa cells prefer to adhere to hbmE cells. Further, it has been suggested that binding between PCa cells and bone marrow endothelial cells is initiated by selectins and stabilized by intergrin binding [139].

Selectins, integrins, and immunoglobulins are known to regulate adhesion between cancer cells and the endothelium [157-161]. Our findings suggested E-Selectin, VCAM-1, and ICAM-1 cell surface abundance was more than doubled in response to increased extracellular calcium. Transient increases in extracellular calcium act as a second messenger
to induce surface expression of VCAM-1 and E-Selectin in human endothelial cells [143]. We further demonstrated that adhesion to C4-2 cells was decreased by 50% in the presence of function blocking antibodies to each adhesion molecule individually. Moss et al., demonstrated that blocking antibodies to E-Selectin and VCAM-1 reduced adhesion of metastatic breast cancer cells to vascular endothelial cells [162]. Multiple studies have reported increased levels of soluble E-Selectin, ICAM-1, and VCAM-1 within the serum of patients with advanced metastatic PCa [163-166] which suggests that these adhesion molecules may have a role in the development of bone metastases. While our observations suggested that E-Selectin, VCAM-1, and ICAM-1 were involved in PCa to hbmE cell adhesion, the mechanisms remain unknown. In addition, there was evidence to suggest that other adhesion molecules are involved in this process because there was no additional decrease in heterotypic cell adhesion when function blocking antibodies were combined nor when saturating doses of function blocking antibodies were used. Further studies need to be conducted to identify other adhesion molecules required for this process such as integrins and other members of the immunoglobulin superfamily.

There has been extensive focus on the effects of extracellular calcium and its role in proliferation and survival of PCa cells [52, 152]. Another mechanism by which extracellular calcium may contribute to the progression of PCa bone metastases is migration. We employed two independent assays to assess cellular motility, transwell migration and wound healing assays. We observed an increase in osteoblastic PCa cell migration when extracellular calcium was used as a chemoattractant. Recently, employing a similar approach, increased extracellular calcium was reported to enhance the migration of breast cancer cells [146]. Our results suggested that extracellular calcium enhanced the general motility of
osteoblastic PCa cells as well as the directional migration; however, these assays were limited because concurrent changes in cell survival and proliferation were not measured. While it is unlikely cells began to proliferate during the 24 hr experiments, this limitation to our studies should not be overlooked.

Invasion is another cellular mechanism that may be required for the colonization of metastastic cancer cells to bone. Employing heterotypic cellular invasion assays, we wanted to identify a role for extracellular calcium in the promotion of PCa cell movement through hbmE cell monolayers. Our observations suggested when extracellular calcium was used as a chemoattractant PCa cell invasion of hbmE cell monolayers was enhanced. Lewalle et al. demonstrated the addition of breast cancer cells to HUVEC monolayers induced a rise in HUVEC intracellular calcium causing the endothelial cells to retract thus allowing breast cancer cells to invade [167]. While we did not measure intracellular calcium changes or the retraction of hbmE cell monolayers, it is reasonable to conclude such changes could have occurred in our studies. Montague et al. demonstrated that treatment of bone marrow endothelial cells with bisphosphonate, a calcium chelating agent, tightened endothelial cell junctions and inhibited transendothelial cell migration of PCa cells [168]; this finding suggests that a rise in intracellular calcium in hbmE cells may alter PCa cell invasion of hbmE cell monolayers.

TRPV6 is a calcium selective ion channel that regulates intracellular calcium. Recent studies have identified TRPV6 ion channel expression to be correlated with PCa progression. We found that in response to transient increases in extracellular calcium, TRPV6, CaSR, and PI3K proteins were significantly enhanced. Recent studies have shown that the CaSR and PI3K have a role in PCa progression [52, 169, 170]. In one study, shRNA
interference of the CaSR decreased skeletal metastases in bone [52]. Activation of the CaSR results in activation of downstream signaling for example the PI3K/AKT signaling pathway. We propose that extracellular calcium activates the CaSR which then increases expression of the CaSR and PI3K. The CaSR and PI3K are responsive to changes in extracellular calcium and since TRPV6 regulates calcium homeostasis, further studies are needed to determine the cellular mechanisms responsible for their increase and if the CaSR and PI3K can regulate TRPV6 ion channel expression. While extracellular calcium regulates TRPV6, it has not been shown to increase channel expression. It is possible that TRPV6 is responsible for the increases in migration and invasion we observed.

This study has investigated the role of extracellular calcium in adhesion, migration, and invasion of osteoblastic PCa cells, in vitro. Our study suggested that hbmE cells can respond to changes in extracellular calcium which enhances osteoblastic PCa cell adhesion. Our initial studies conclude that E-Selectin, ICAM-1, and VCAM-1 are involved in this adhesion process but additional studies are necessary to determine other adhesion molecules involved. We also demonstrated that calcium can enhance PCa cell migration and invasion of hbmE cell monolayers. The CaSR, PI3K and TRPV6 protein expression were enhanced by transient elevations in extracellular calcium which suggests that these proteins may be involved in the increased PCa cell migration and invasion we observed. This study provided evidence to suggest that changes in calcium concentrations in vivo may act upon bone marrow endothelial cells as well as osteoblastic PCa cells to enhance PCa cell colonization to bone.
CHAPTER 3:

TRPV6 Ion Channels Modulate Human Metastatic Prostate Cancer Cell Invasion and Adhesion
3.1 Abstract

We have previously reported that intermittent PTH 1-34 enhances the colonization of human osteoblastic PCa cells to murine bone. While the mechanism is unknown, we propose that transient increases in serum calcium following PTH 1-34 administration, confer a metastatic advantage to circulating PCa cells, signaled, in part, via TRPV6 ion channels. We tested the hypothesis that TRPV6 ion channels are required for PCa cell metastatic potential, in vitro. To test our hypothesis, shRNA lentiviral particles were used to decrease TRPV6 expression in C4-2 PCa cells. Knockdown was confirmed by qPCR, Western blot analysis, and immunocytochemistry. Proliferation, transwell migration, and heterotypic cell adhesion assays were employed to evaluate the metastatic potential of C4-2 cells with reduced levels of TRPV6 relative to WT and control shRNA cells. QPCR and Western blot analysis were used to identify several clones that expressed reduced TRPV6 protein (44-63%). Confocal micrographs of cells probed with TRPV6 specific antibodies, confirmed TRPV6 localization in clones was reduced at the cell surface. Relative to control cells, cells with decreased TRPV6 expression had a slower rate of proliferation. Heterotypic cell adhesion assays demonstrated that cells with reduced TRPV6 expression were less adherent (34%) to hbmE cells. Pretreatment of hbmE cells with 2.5 mM CaCl₂ enhanced adhesion of control cells by 78%; however, this response was abolished in TRPV6 knockdown clones. Transwell invasion assays suggested that increased extracellular calcium [2.5 mM CaCl₂] enhanced invasion of WT and control cell lines; however, relative to controls, invasion was reduced by 57% in knockdown clones. Our observations support our hypothesis and suggest, for the first time, that TRPV6 may serve an important role in modulating metastatic PCa invasion and adhesion to bone marrow endothelial cells, in vitro.
3.2 Introduction

Calcium is a pivotal signaling ion essential in regulating proliferation, differentiation, and survival of normal and malignant cells [104]. Calcium acts as a second messenger in multiple intracellular signaling pathways which govern cell homeostasis and physiopathology [109]. Recent studies have shown that increases in intracellular calcium enhance cancer cell proliferation, adhesion, and migration [52, 130, 146]. Intracellular calcium levels are tightly regulated by ion pumps, calcium binding proteins, and plasma membrane ion channels [171].

Transport of extracellular calcium across epithelial plasma membranes is mediated in part by a superfamily of ion channels, Transient Receptor Potential (TRP) channels which have an important role in regulating calcium homeostasis [109]. TRPV6 was first identified in the intestine and kidney where it regulates calcium entry [113]. Calcium entry through TRPV6 is a rate limiting step tightly controlled by intracellular calcium levels [99, 103, 108, 115]. TRPV6 expression is regulated by hormones, primarily 1,25(OH)2D3, estrogen, and androgens [123, 171]. Recently, the TRP family member Vanilloid 6 (V6), a highly selective calcium ion channel, has been reported to be expressed in advanced PCa [107]. TRPV6 is undetectable in benign prostate tissue [107]; however, Fixemer and colleagues analyzed prostate tissue biopsy samples from locally advanced tumors and lymph node metastases and found that TRPV6 ion channel expression positively correlated with Gleason score [126]. TRPV6 expression was reduced by a minimum of 50% in a non-metastatic PCa cell line, LNCaP, using siRNA which resulted in a decrease in proliferation and an increase in the number of apoptotic cells [127] suggesting TRPV6 ion channels may have a role in PCa cell
cycle progression. The role of TRPV6 ion channels in the development of PCa bone metastases has not been investigated.

We have previously reported that intermittent PTH 1-34 administration enhanced the colonization of human osteoblastic PCa cells to murine bone [74]. While the mechanism remains unclear, a single injection of PTH 1-34 transiently increases serum calcium levels [94, 95]. Elevated serum calcium levels have been correlated to an increased risk of developing fatal PCa [93, 97]. Although it is not known how serum calcium levels modulate PCa cell behavior. We propose that transient increases in serum calcium confer a metastatic advantage to circulating PCa cells, signaled, in part, via TRPV6 ion channels. We tested the hypothesis that TRPV6 ion channels are required for PCa cell metastatic potential, in vitro. To test our hypothesis, shRNA lentiviral particles were employed to knock down TRPV6 expression in C4-2 PCa cells. We found that cells with reduced levels of TRPV6 demonstrated decreased proliferation, altered adhesive and invasive properties to hbmE cell monolayers, and did not respond to increases in extracellular calcium compared to control cells. Together our observations suggest TRPV6 may serve an important role in modulating the metastatic potential of osteoblastic PCa cells, particularly invasion and adhesion to bone marrow endothelial cells, in vitro.

3.3 Materials and Methods

3.3.1 Cell Lines and Culture

Human C4-2 PCa cell lines, kindly provided by Dr. Robert A. Sikes (University of Delaware), were cultured in T-Medium (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO₂. Cells were sub-cultured when 80-90% confluent using
0.05% (v/v) trypsin/EDTA. All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. All experiments were completed using cells between passages 16-25.

Human bone marrow endothelial cells (hbmE), provided by Dr. G Almeida-Porada (University of Nevada, Reno) were cultured in M199 medium supplemented with endothelial cell growth supplement (R&D systems Inc., Minneapolis, MN), 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin, 2 mM glutamax and heparin (15 U/ml). Cells were sub-cultured when 80-90% confluent using 0.05% (v/v) trypsin/EDTA. All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. All experiments were completed using cells between passages 5-10.

3.3.2 ShRNA Transduction

C4-2 cells (100,000) were seeded in 6 well dishes and when 50% confluent were treated with 1 or 2 infectious units/cell of TRPV6 shRNA lentiviral particles (Santa Cruz, Santa Cruz, CA) in T-Medium supplemented with 4 µg/ml polybrene overnight. The medium was changed the following day, 15 clones were selected, placed in 24 well dishes, and grown under selective pressure using 0.25 µg/ml puromycin dihydrochloride. Control shRNA lentiviral particles, encoding a scrambled shRNA sequence, were used in conjunction. Knockdown of TRPV6 was confirmed by qPCR, Western blot, and immunocytochemistry.

3.3.3 Proliferation Assay

Cells (5,000) were seeded in 96 well plates in T-Medium (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) serum and allowed to adhere. Every 24 hrs over a period of 6 days, cell density was quantified using a CyQuant Proliferation Assay (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.
3.3.4 Heterotypic Cell Adhesion Assay

HbmE cells (80,000) were seeded in 48 well tissue culture dishes and allowed to become confluent monolayers. On the day of the experiment, hbmE monolayers were treated with 1.25 mM CaCl₂ for 3 hrs. C4-2 cells expressing basal and reduced levels (44-63%) of TRPV6 were washed with PBS then removed with 5 mM EDTA and labeled with 25 µM of Calcein-AM and placed on top of hbmE cell monolayers for a period of 3 hrs. Cells were then washed with PBS, gently agitated to remove un-adherent cells, and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence intensity (485/515 nm). The sensitivity of this assay was predetermined by creating a standard curve employing known numbers of Calcein-AM labeled cells.

3.3.5 Migration Assay

Calcium and serum starved (~12 hrs) C4-2 cells expressing basal and reduced levels (44-63%) of TRPV6 were detached from culture surfaces with 5 mM EDTA, re-suspended in calcium free DMEM, and labeled with 25 µM of Calcein-AM (Invitrogen, Carlsbad, CA) for 20 min. Subsequently, cells were washed twice with calcium free DMEM (Invitrogen) and 150,000 labeled cells were seeded on the top well of a tissue culture insert (8 micron pore, BD Falcon, San Jose, CA) in serum and calcium free DMEM. The bottom of the well contained 2.50 mM CaCl₂ as a chemoattractant. After 24 hrs, tissue culture inserts were placed in 0.05% (v/v) trypsin/EDTA for 20 min to remove migrated cells from the bottom of the insert. Migrated cells were quantified by measuring fluorescence intensity (485/515 nm). The sensitivity of this assay was predetermined by creating a standard curve employing known numbers of Calcein-AM labeled cells.
3.3.6 Invasion Assay

Invasion assays were performed similarly to migration assays but prior to seeding C4-2 cells, 50,000 hbmE cells were seeded in the top well of the tissue culture insert to create confluent monolayers. Calcium and serum starved (~12 hrs) C4-2 cells expressing basal and reduced levels (44-63%) of TRPV6 were detached from culture surfaces with 5 mM EDTA, re-suspended in calcium free DMEM, and labeled with 25 µM of Calcein-AM for 20 min. Subsequently, cells were washed twice with calcium free DMEM and 150,000 labeled cells were seeded on the top of the hbmE cell monolayers in serum and calcium free DMEM. The bottom of the well contained 2.50 mM CaCl₂ as a chemoattractant. After 24 hrs, tissue culture inserts were placed in 0.05% (v/v) trypsin/EDTA for 20 min to remove cells from the bottom of the insert. Cells removed from the bottom of the insert were quantified by measuring fluorescence intensity (485/515 nm).

3.3.7 Western blot Analysis

Total protein was isolated from C4-2 cells (300,000) using lysis buffer containing: 10 mM Tris–HCl (pH 7.4), 140 mM NaCl, 0.2% (v/v) Triton X-100 and 7X protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were centrifuged at 10,000 G at 4°C for 7 min. Total protein (40 µg) was loaded into each well of a 7.5% (w/v) SDS-Page gel, electrophoresed, and transferred to a nitrocellulose membrane by semi-dry Western blotting (Bio-Rad laboratories, Redmond, WA). The membrane was blocked in 5% (v/v) BSA in 0.05% (v/v) PBST for 45 min then probed overnight using antibodies specific for TRPV6 (1:500, Alomone, Jerusalem, Israel), CaSR (1:500, Abcam, Cambridge, MA), PI3K (1:500, Cell Signaling, Danvers, MA), CD44 (1:200, Santa Cruz, Santa Cruz, CA) and GAPDH (1:10000, Genscript, Piscataway, NJ). Goat anti-rabbit (Bio-Rad laboratories, Redmond,
WA) and NeutraAvidin (Thermo Scientific, Rockville, IL) secondary antibodies conjugated to HRP were used, respectively. The bands on the membrane were visualized using enhanced chemiluminescence (Pierce Biotechnologies, Rockville, IL). Densitometric analysis was performed using a Bio-Rad acquisition system and data are expressed mean IOD relative to GAPDH.

3.3.8 RNA Extraction and Quantitative PCR

Trizol reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA from C4-2 cells (100,000). Total RNA (500 ng) was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), RNA was denatured at 42°C for 50 min, and 200 U of SuperScript II reverse transcriptase enzyme was used in a final reaction volume of 20 µl. One micro-liter of cDNA was used for qPCR using SYBR Green (Applied Biosystems, Foster City, CA) and primers specific for TRPV6 and 18S (Table 2.1). Sequences were created using the National Center for Biotechnology Information’s basic local alignment search tool. Sequences were then validated by qualitative PCR. Quantitative PCR was carried out using the RotorGene Real-Time DNA amplification system (Corbett Research, Sydney, Australia). Gene expression was normalized to 18S.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>TRPV6</td>
<td>ATGGTGATGCGGCTCATCAGTG</td>
<td>GTAGAAGTGCCCTAGCTCTCG</td>
</tr>
<tr>
<td>18S</td>
<td>GAGAAACGGCTACC</td>
<td>CACCAGACTTGGCCC</td>
</tr>
</tbody>
</table>

Table 3.1 Forward and Reverse primer sequences for Real Time PCR.

3.3.9 Immunocytochemistry

Cells were grown on glass coverslips, washed with PBS, and fixed with 2% (v/v) paraformaldehyde (15 min) in PBS. The cells were then washed with PBS, incubated with
3% (v/v) BSA (30 min), and subjected to the conventional indirect immunostaining procedure using antibodies specific to TRPV6 (1:500, Alomone, Jerusalem, Israel). Goat anti-rabbit IgG conjugated to AlexaFluor 594 (1:1100, Molecular Probes, Carlsbad, CA) was used as a secondary antibody. Images of cells were captured with a 40X objective lens using an Olympus Fluo View (FV1000) confocal microscope with brightness set at 670 HV and 7% laser power. Control cells were incubated with only secondary antibodies.

3.3.10 Statistical Analysis

All experiments were conducted in triplicate and repeated at least three times. Graphpad PRISM version 5.0 (GraphPad Software, San Diego CA) was used for statistical analysis. One-way ANOVA with a Bonferroni post hoc test was performed with experiments containing multiple groups to compare all columns of data. Two-way ANOVA was used to analyze statistical significance of proliferation assays. Two tailed t-tests were used where appropriate. All data are expressed as mean ± SD and were considered significant if \( p \leq 0.05 \).

3.4 Results

3.4.1 TRPV6 Knockdown

Lentiviral shRNA particles were employed to decrease TRPV6 expression in C4-2 cells. Fifteen clones (TRP1-15) were selected and screened for TRPV6 expression using qPCR, Western blot analysis, and immunocytochemistry. Control shRNA lentiviral particles, encoding a non-specific scrambled sequence, were also used and 15 control clones (C1-15) were also selected and screened. Western blotting identified four clones (TRP-9,11,12,13) with reduced TRPV6 expression (44-63%) compared to WT cells which were chosen to further characterize. One control clone (C2.6) was also selected for further characterization.
because it expressed similar levels of TRPV6 protein compared to WT cells (Figure 3.1). Knockdown was also validated by qPCR (Figure 3.2) and immunocytochemistry (Figure 3.3).

### 3.4.2 Characterization of Clones

Clones were functionally characterized by measuring changes in proliferation, adhesion, invasion and their response to extracellular calcium. Changes in proliferation were measured using CyQuant Dye Proliferation assay. All clones with decreased levels of TRPV6 expression had reduced rates of growth that plateaued after 96 hrs relative to both WT and Control shRNA cells. Control shRNA cell proliferation was similar to WT cell proliferation (Figure 3.4).

Heterotypic cell-to-cell adhesion assays were employed using cells with decreased TRPV6 expression and hbmE cells. Adhesion was decreased (26-45%) in all clones with reduced TRPV6 expression relative to WT and Control shRNA cells. Control shRNA cell adhesion to hbmE cell monolayers was similar to WT cell adhesion (Figure 3.5). When hbmE cell monolayers were exposed to CaCl$_2$ [1.25 mM] for 3 hrs prior to the addition of C4-2 cells, WT and control shRNA C4-2 cells exhibited increased adhesion to hbmE cell monolayers. Interestingly, C4-2 cells with reduced levels of TRPV6 expression did not increase adhesion in response to hbmE cells treated with CaCl$_2$ (Figure 3.6).

To identify why cells with decreased TRPV6 expression did not increase adhesion to hbmE cells treated with CaCl$_2$, Western blot analysis was conducted to measure CD44 protein expression. CD44 is a ligand for E-Selectin which may be involved in the adhesion process between hbmE cells and PCa cells [172, 173]. Moreover, our previous observations suggested that E-Selectin cell surface expression on hbmE cells was enhanced by exposure to CaCl$_2$ and that function blocking antibodies to E-Selectin reduced C4-2 to hbmE cell
adhesion (unpublished observations). We observed a significant decrease (53-58%) in CD44 protein expression in cells with reduced levels of TRPV6 expression compared to WT and control shRNA cells (Figure 3.7).

Migration assays conducted using CaCl2 [2.50 mM] as a chemoattractant identified reduced migration (45%) in only one clone with decreased TRPV6 expression (TRP-12); interestingly this clone also has the highest percentage of TRPV6 knockdown (63%) (Figure 3.8). Invasion assays were conducted to identify changes in the ability of cells with reduced TRPV6 expression to invade hbmE cell monolayers. Cells with reduced TRPV6 expression poorly invaded hbmE cell monolayers when CaCl2 was used as a chemoattractant, invasion was decreased by 35-67% compared to WT and Control shRNA cells (Figure 3.9).

To identify the response to increases in extracellular calcium in clones with reduced TRPV6 expression at the protein level, cells were treated with CaCl2 [2.50 mM] for 3 hrs and total protein was isolated 24 hrs after treatment. Western blot analysis suggested TRPV6, CaSR, and PI3K protein expression was increased in WT and control shRNA cells following exposure to CaCl2. In contrast, there were no observed differences in TRPV6 (Figure 3.10), CaSR (Figure 3.11), or PI3K (Figure 3.12) protein in cells with reduced TRPV6 expression following exposure to CaCl2.
Figure 3.1 Lentiviral particles reduced TRPV6 protein expression in C4-2 cells (44-63%). Total protein was isolated from wild type (WT), control shRNA clone (Control), and knockdown clones (TRP-9, TRP-11, TRP-12, TRP-13) and Western blot analysis was conducted. Membranes were probed with antibodies specific for TRPV6. The top panel is comprised of representative Western blot images of TRPV6 and GAPDH for each cell type with molecular weight denoted on the right. The bars represent the mean IOD +/- SD of triplicate observations of TRPV6 protein relative to GAPDH for each cell type. * indicates significantly different from WT and Control, p≤0.05.
Figure 3.2 Lentiviral particles reduced TRPV6 steady state mRNA expression in C4-2 cells. Total RNA was extracted from C4-2 cells and qPCR was conducted using primers specific to TRPV6 and 18S. The bars represent the mean expression of TRPV6 mRNA relative to 18S +/- SD for triplicate observations for wild type (WT), control shRNA clone (Control), and knockdown clones (TRP-9, TRP-11, TRP-12, TRP-13). * indicates significantly different from WT and Control, p≤0.05.
Figure 3.3 Lentiviral particles reduced TRPV6 localization in C4-2 cells. C4-2 cells were grown on glass coverslips, fixed with 2% paraformaldehyde, and then probed with antibodies specific to TRPV6. Goat anti-rabbit IgG conjugated to AlexaFluor 594 (1:1100) was used as secondary antibodies. Images were captured using an Olympus Fluo View (FV1000) confocal microscope. Confocal images represent TRPV6 (red) localization and DAPI (blue) nuclear stain for wild type (WT), control shRNA clone (Control), and knockdown clones (TRP-9 and TRP-12).
Figure 3.4 Cellular proliferation was reduced in C4-2 cells with decreased TRPV6 expression. Proliferation of wild type (WT), control shRNA (Control), and knockdown clones (TRP-9, TRP-11, TRP-12) were quantified every 24 hrs over 144 hr period by CyQuan ant dye. Each point represents the mean fluorescence +/- SD of triplicate observations. * indicates significantly different from WT and Control, p≤0.05.
Figure 3.5 C4-2 cells with reduced TRPV6 expression poorly adhere to hbmE cell monolayers. Calcein-AM labeled C4-2 cells with reduced levels of TRPV6 (TRP-11 and TRP-12) were added to hbmE cell monolayers. After 3 hrs, the wells were washed with PBS, gently agitated to remove un-adherent cells, and lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence intensity (485/515 nm). The bars represent the mean fluorescence +/- SD of triplicate observations adherent C4-2 cells. * indicates significantly different from WT and Control, p≤0.05.
Figure 3.6 Calcium pretreatment of hbmE cell monolayers did not enhance adhesion of C4-2 cells with reduced TRPV6 expression. HbmE cells were exposed to CaCl$_2$ [1.25 mM] for 3 hrs then C4-2 cells loaded with Calcein-AM were added to hbmE cell monolayers. After 3 hrs, wells were washed with PBS, gently agitated to remove un-adherent cells and then lysed with 5% (v/v) NP-40. Adherent C4-2 cells were quantified by measuring fluorescence intensity (485/515 nm). The bars represent the mean fluorescence +/- SD of adherent WT cells added to untreated hbmE cell monolayers (white), WT cells added to CaCl$_2$ treated hbmE cell monolayers (green), control shRNA cells added to untreated hbmE cell monolayers (checkered), control shRNA cells added to CaCl$_2$ treated hbmE cell monolayers (green checkered), C4-2 cells with reduced levels of TRPV6 (TRP-11, TRP-12) added to untreated hbmE cell monolayers (gray bars), and C4-2 cells with reduced levels of TRPV6 (TRP-11, TRP-12) added to CaCl$_2$ treated hbmE cell monolayers (striped green bars). * indicates significantly different from untreated controls, p≤0.05.
Figure 3.7 CD44 expression was decreased in C4-2 cells with reduced TRPV6 expression. Total protein was isolated from wild type (WT), control shRNA (Control), and knockdown clones (TRP-11 and TRP-12) and analyzed by Western blotting. Membranes were probed with antibodies specific to CD44 and GAPDH (loading control) and expression levels were compared to WT and Control cells. The top panel is a representative Western blot image of CD44 and GAPDH for each cell type. The bars represent the mean IOD +/- SD of triplicate observations of relative CD44 expression to GAPDH for each cell type. * indicates significantly different from WT and Control, p≤0.05.
Figure 3.8 Migration was decreased when TRPV6 expression was reduced by at least 63%. Calcium and serum starved (~12 hrs) C4-2 cells (WT, Control shRNA, TRP-11, and TRP-12) were loaded with 25 μM of Calcein-AM and placed in the top well of a tissue culture insert in serum and calcium free DMEM. The bottom of the well contained 2.5 mM CaCl$_2$ as a chemoattractant. After 24 hrs, cells on the bottom of the insert were removed with 0.05% (v/v) trypsin/EDTA and quantified by measuring fluorescence intensity (485/515 nm). Each bar represents the mean fluorescence +/- SD of triplicate observations of cells isolated from the bottom of the insert. * indicates significantly different from WT and Control, p≤0.05.
**Figure 3.9** C4-2 cells expressing low levels of TRPV6 poorly invaded hbmE cell monolayers. HbmE cell monolayers seeded on the top of tissue culture inserts were overlaid with calcium and serum starved (~12 hrs) Calcein-AM labeled C4-2 cells (WT, Control shRNA, TRP-11, and TRP-12). The bottom of the well contained 2.5 mM CaCl₂ as a chemoattractant. After 24 hrs, cells on the bottom of the insert were removed with 0.05% (v/v) trypsin/EDTA and quantified by measuring fluorescence intensity (485/515 nm). Each bar represents the mean fluorescence +/- SD of triplicate observations of C4-2 cells on the bottom of the insert.* indicates significantly different from WT and Control cells, p≤0.05.
Figure 3.10 Transient elevations in CaCl$_2$ did not enhance TRPV6 expression in C4-2 cells with reduced levels of TRPV6 expression. C4-2 cells (WT, Control shRNA, TRP-11, and TRP-12) were exposed to CaCl$_2$ for 3 hrs (Green bars) and total protein was isolated after 24 hrs. Western blot analysis was conducted and membranes were probed with antibodies specific to TRPV6 and GAPDH. The bars represent the mean IOD +/- SD of triplicate observations of TRPV6 relative to GAPDH in WT cells (white), WT cells exposed to CaCl$_2$ (green), control shRNA cells (checkered), control shRNA cells exposed to CaCl$_2$ (green checkered), C4-2 cells with reduced levels of TRPV6 (TRP-11, TRP-12; gray bars), and C4-2 cells with reduced levels of TRPV6 (TRP-11, TRP-12) exposed to CaCl$_2$ (striped green bars) * indicates significantly different from untreated controls, p≤0.05.
Figure 3.11Transient elevations in CaCl\(_2\) did not enhance CaSR expression in C4-2 cells with reduced levels of TRPV6 expression. C4-2 cells (WT, Control shRNA, TRP-11, and TRP-12) were exposed to CaCl\(_2\) for 3 hrs (Green bars) and total protein was isolated after 24 hrs. Western blot analysis was conducted and membranes were probed with antibodies specific to CaSR and GAPDH. The bars represent the mean IOD +/- SD of triplicate observations of CaSR relative to GAPDH in WT cells (white), WT cells exposed to CaCl\(_2\) (green), control shRNA cells (checkered), control shRNA cells exposed to CaCl\(_2\) (green checkered), C4-2 cells with reduced levels of TRPV6 (TRP-11, TRP-12; gray bars), and C4-2 cells with reduced levels of TRPV6 (TRP-11, TRP-12) exposed to CaCl\(_2\) (striped green bars) * indicates significantly different from untreated controls, p≤0.05.
Figure 3.12 Transient elevations in CaCl$_2$ did not enhance PI3K expression in C4-2 cells with reduced levels of TRPV6 expression. C4-2 cells (WT, Control shRNA, TRP-11, and TRP-12) were exposed to CaCl$_2$ for 3 hrs (Green bars) and total protein was isolated after 24 hrs. Western blot analysis was conducted and membranes were probed with antibodies specific to PI3K and GAPDH. The bars represent the mean IOD +/- SD of triplicate observations of PI3K relative to GAPDH in WT cells (white), WT cells exposed to CaCl$_2$ (green), control shRNA cells (checkered), control shRNA cells exposed to CaCl$_2$ (green checkered), C4-2 cells with reduced levels of TRPV6 (TRP-11, TRP-12; gray bars), and C4-2 cells with reduced levels of TRPV6 (TRP-11, TRP-12) exposed to CaCl$_2$ (striped green bars) * indicates significantly different from untreated controls, p≤0.05.
3.5 Discussion

TRPV6 ion channels have previously been linked to PCa tumor progression and are thought to promote PCa cell growth and survival [107, 127]. Prior to this study, the role of TRPV6 ion channels in osteoblastic PCa cells had not been investigated. This study was carried out to identify a role for TRPV6 ion channels in the metastatic potential of the C4-2 PCa cell line. We transduced C4-2 cells with TRPV6 shRNA lentiviral particles to produce cells which had a stable reduction in TRPV6 protein expression. The maximum percentage of knockdown attained using this method was 63%. Cells with decreased TRPV6 expression had slower rates of proliferation, decreased heterotypic cell adhesion, a diminished ability to migrate, and poorly invaded hbmE cell monolayers. This is the first report to suggest TRPV6 ion channel expression impacts the adhesive and invasive abilities of C4-2 cells, in vitro.

Schwarz et al. reported that HEK-293 cells transfected with TRPV6 had an increased rate of proliferation [175]. It was concluded that the increase in proliferation observed was due to a slight increase [< 20 nM] in intracellular calcium [175]. In contrast, LNCaP cells (the parental cell line to C4-2 cells) engineered to express reduced levels of TRPV6 exhibited decreased proliferation [127]. This is consistent with our observations that C4-2 cells with decreased TRPV6 expression proliferated at a slower rate relative to controls. Previous reports suggest that PCa cell proliferation was dependent on intracellular calcium entry which subsequently activated nuclear factor of activated T-cell (NFAT) transcription factor [176]. LNCaP cells co-transfected with siRNA-TRPV6 and pNFAT luciferase plasmid, suggested that TRPV6 induced calcium entry activated the calcium promoter on the NFAT transcription factor [127]. It is likely that TRPV6 also mediates intracellular calcium entry in C4-2 cells which subsequently activates NFAT; however, studies directed to answer this
question have not been conducted. While we did not directly measure intracellular calcium levels, we speculate that C4-2 cells with reduced levels of TRPV6 ion channels have low intracellular calcium levels since TRPV6 ion channels are primarily responsible for regulating calcium homeostasis [109].

Alterations in intracellular calcium have been reported to regulate adhesion and migration of cancerous and noncancerous cells [52, 104, 130, 146]. A recent investigation reported an increase in TRPV6 expression in uterine endometrium during embryo implantation [177]. This suggests that TRPV6 may have a role in adhesion primarily because during implantation many cellular adhesion molecules increase expression to ensure the implantation between the embryo and the endometrium occurs [177]. Herein, we presented data suggesting that TRPV6 had a role in heterotypic cell adhesion. Cells expressing reduced TRPV6, at least by 44%, poorly adhere to hbmE cell monolayers. Further, WT cell adhesion to hbmE cell monolayers was enhanced when hbmE cells were pretreated with CaCl₂; however, cells with reduced levels of TRPV6 did not increase adhesion to hbmE cell monolayers pre-exposed to CaCl₂, suggesting TRPV6 expression is related to adhesion molecules present on osteoblastic PCa cells.

CD44 is one potential adhesion molecule necessary for PCa cell adhesion to hbmE cell monolayers [172, 173]. Recently, CD44 isolated from metastatic breast cancer cells was reported to express multiple binding sites for E-Selectin [178]. Zen et al. further demonstrated that CD44 mediated cellular adhesion and migration by interacting with E-Selectin. CD44 has not been linked to TRPV6 previously; however, our investigation suggests that TRPV6 expression may directly or indirectly regulate CD44 expression and/or its’ presence at the cell membrane. CD44 expression in TRPV6 knockdown clones was
reduced and this could explain why cells with reduced levels of TRPV6 poorly adhered to hbmE cell monolayers.

Our proposed mechanism in which TRPV6 could effect CD44 expression is through the S100A10-annexin 2 complex. S100A10 is a calcium binding protein that commonly binds annexin-2, a phospholipid binding protein [179]. S100A10 is necessary for trafficking TRPV6 to the plasma membrane [116, 180]. S100A10 interacts with TRPV6 through the C-terminal intracellular tail and binding S100A10 is necessary for TRPV6 to function properly [116]. S100A10 forms a heterotetramer complex with annexin-2 which is also required for TRPV6 translocation from the cytosol to the plasma membrane [116, 180]. CD44 co-localizes with Annexin-2 within the plasma membrane of epithelial cells [181], however, it is unknown whether this interaction occurs in PCa cells. While further studies are necessary to confirm these interactions and relationships, our model suggests that when TRPV6 expression is decreased, less S100A10-Annexin-2 complexes are present within the plasma membrane which results in a reduction of CD44 present at the cell surface and a decrease in overall adhesion.

TRP ion channel expression affects intracellular calcium levels [182] which ultimately alters cellular processes involved in cancer progression for example, migration and invasion [183-185]. We found that cells with decreased TRPV6 expression poorly invaded hbmE cell monolayers. TRPV6 has recently been reported to be highly expressed during the implantation phase of human and porcine embryos, we posit that TRPV6 has a role in invasion because invasion of the endometrium is required for implantation [177, 186]. We also performed transwell migration assays using calcium as a chemoattractant and found that only one clone demonstrated reduced migration. Interestingly, this clone also had the largest
percentage of TRPV6 knockdown (63%), suggesting that there may be a threshold of knockdown required for the disruption of migration. TRPV1, another TRP family member has been linked to cellular migration; one study demonstrated that activation of TRPV1 enhanced migration of human hepatoblastoma cells, \textit{in vitro} [187]. However, studies that have investigated TRP channels and their role in migration are limited. Future studies should investigate other metastatic cancer cell lines to confirm that TRPV6 modulates migration and invasion.

Multiple studies have demonstrated that G-protein coupled receptors are responsible for activating TRPV6 [125, 127, 129]. We have demonstrated that TRPV6, CaSR, and PI3K protein expression was enhanced in response to increased extracellular calcium. In contrast, cells with decreased TRPV6 expression did not increase TRPV6, CaSR, or PI3K protein expression in response to increased extracellular calcium. The inability of these cells to respond to increases in extracellular calcium suggests that the cells’ ability to handle calcium had been disrupted. It also suggests that the CaSR and PI3K may be connected to TRPV6 signaling, although future studies are necessary to confirm this link.

In conclusion, the current study is the first to stably decrease TRPV6 expression in an osteoblastic PCa cell line. Our observations suggest that TRPV6 ion channels are necessary for the proliferation, adhesion, and invasion of osteoblastic PCa cells. Further, we provide evidence to suggest that TRPV6 ion channel expression is linked to CD44, a potential adhesion molecule. Although these observations, together, support our hypothesis, further investigation to identify specific cellular mechanisms responsible are warranted. This study provides evidence to suggest that TRPV6 ion channel expression is correlated with PCa progression.
Chapter 4:

TRPV6 Ion Channels and Prostate Cancer Cell Early Colonization to Murine Bone
4.1 Abstract

We have previously reported that intermittent PTH 1-34 administration enhances the colonization of human osteoblastic PCa cells to murine bone. In this study, we utilize our animal model to test the hypothesis that TRPV6 ion channels are required for the early colonization of PCa cells to murine bone. To test our hypothesis, shRNA lentiviral particles were employed to knock down TRPV6 expression in C4-2 prostate cancer cells (~60-63%). SCID/Beige mice were administered intermittent PTH 1-34 and injected with PCa cells with reduced levels of TRPV6 expression. Eight weeks post injection, serum was collected and long bones and lumbar vertebrae were harvested for analysis. Bone tissue sections were stained with antibodies specific to prostate specific antigen (PSA) and pan-cytokeratin to detect PCa cells within the bone marrow. PSA and pan-cytokeratin positive cells were visualized in the long bones of 100% of animals administered PTH 1-34 and injected with WT C4-2 cells compared to 20% of vehicle treated animals; these observations are consistent with our previous findings. Similar results were observed for animals injected with control shRNA cell lines. In contrast, PSA and pan-cytokeratin positive cells were localized in the long bones of only 20% and 60% of animals administered PTH 1-34 and injected with TRP-11 or TRP-12 clones, respectively. This is the first report employing shRNA to stably reduce TRPV6 expression in a human osteoblastic PCa cell line, *in vivo*. Our limited observations support our hypothesis and suggest that TRPV6 may have a role in the early colonization of PCa cells to murine bone.

4.2 Introduction

Prostate cancer is the most common disease and second leading cause of cancer related deaths in North American men [1-3]. In 2011, the National Cancer Institute estimated
approximately 34,000 reported deaths related to PCa in the United States. About 60-85% of men with advanced PCa have associated bone metastases [2]. PCa bone metastases contribute to bone pain and an increased risk of fracture which can potentially lead to paralysis due to spinal cord compression [7]. There are currently no effective therapeutic treatments available to prevent or fully eradicate PCa bone metastases [1, 8]. A better understanding of how and why PCa cells colonize bone is critical to develop novel therapeutics to treat and prevent skeletal metastases.

Recently, calcium has emerged as having a role in PCa progression. Calcium is a pivotal signaling ion that is essential in regulating proliferation, differentiation, and survival of normal and malignant cells [52, 104, 130, 146]. Calcium acts as a second messenger in multiple intracellular signaling pathways which govern cell homeostasis and physiopathology [109]. A number of recent studies have shown that extracellular calcium enhances cancer cell proliferation, adhesion, and migration [52, 130, 146]. It is not known whether changes in extracellular calcium can enhance PCa cell progression, in vivo. Intracellular calcium levels are tightly regulated by ion pumps, calcium binding proteins, and plasma membrane ion channels [171].

Transport of calcium across epithelial plasma membranes is mediated in part by a superfamily of ion channels, Transient Receptor Potential (TRP) channels which have a role in regulating calcium homeostasis [109]. Recently, the TRP family member Vanilloid 6 (V6), a highly selective calcium ion channel, has been reported to be expressed in advanced PCa [107]. TRPV6 was first identified in the intestine and kidney where it regulates calcium entry [113]. Calcium entry through TRPV6 is regulated by intracellular calcium levels [110, 114, 119, 121]. TPRV6 channel expression is controlled by hormones, primarily 1,25(OH)2D3,
estrogen, and androgens [123, 171]. There have only been a handful of studies to investigate the role of TRPV6 in PCa progression [107, 126, 127]. TRPV6 is undetectable in benign prostate tissue [107]; however, Fixemer and colleagues analyzed prostate tissue biopsy samples from locally advanced tumors and lymph node metastases and concluded TRPV6 ion channel expression was positively correlated with Gleason score [126]. The function of TRPV6 in non-metastatic PCa cell lines has been investigated and although the mechanisms have not been fully identified, TRPV6 was suggested to have a role in PCa cell proliferation and survival [127]. Nonetheless, a role for TRPV6 ion channels in other aspects of PCa metastasis has not been investigated; specifically its role in the colonization of PCa cells of the bone microenvironment.

In this study, we tested the hypothesis that TRPV6 ion channels were required for the colonization of PCa cells to murine bone. To test this hypothesis, SCID/Beige mice were pre-administered PTH 1-34 to enhance bone turnover and osteoblastic PCa cell uptake in bone [74, 85]; and animals were subsequently inoculated with C4-2 cells engineered to express reduced levels of TRPV6. Skeletal tissue sections including long bones and lumbar vertebrae were analyzed 8 weeks post-inoculation by immunohistochemistry. This analysis suggested that one clone, expressing reduced levels of TRPV6, poorly developed micrometastases in the long bones of animals administered PTH compared to animals inoculated with WT C4-2 cells. In contrast, there were no differences identified in the localization of PCa epithelial cells in the lumbar vertebrae. Our observations suggest TRPV6 may have a role in the early colonization of osteoblastic PCa cells to murine bone.
4.3 Materials and Methods

4.3.1 Materials

Human PTH 1-34 was purchased from Bachem Inc. (Torrance, CA). Polyclonal antibodies for wide spectrum screening of pan-cytokeratin and PSA were purchased from Dako North America, Inc. (Carpinteria, CA), and BioGenex (San Ramon, CA), respectively. Enzyme-Linked Immunosorbent Assays (ELISA) for PSA were purchased from Anogen Inc. (Mississauga, Ontario, Canada). Unless otherwise stated, all other reagents were purchased from Sigma Chemicals, Co (St. Louis, MO).

4.3.2 Preparation of PTH

PTH 1-34 was reconstituted in 10 mM acetic acid/sterile PBS solution and stored at -80°C at a concentration of 400 μg/ml in siliconized tubes. For injections, the stock solution was diluted to 40 μg/ml in sterile PBS. Control animals were injected with equivalent volumes of acetic acid diluted in PBS [74, 85].

4.3.3 Animals

Forty eight, six week old, male, C.B-17 SCID/Beige mice were purchased from Taconic Laboratories (Rockville, MD), and stabilized in the animal research facility at the Penn State College of Medicine. Mice were housed 5 per cage, maintained under specific pathogen-free conditions, in a continuously HEPA filtered room, maintained between 21-22°C, with 40-60% humidity on 12 hr light and dark cycles and given free access to food and water. Animal body weights were recorded before the start and then every other day during the experiment [74, 85].
4.3.4 Experimental Design

At 8 weeks of age, mice were divided into 8 groups of equal average body weight. Groups 1-4 (n=6/group) served as treatment groups and were administered intermittent doses of PTH 1-34 (40 μg/kg). Groups 5-8 (n=6/group) served as vehicle control animals and were administered equivalent amounts of acetic acid diluted in PBS. All injections were administered subcutaneously, once per day for five consecutive days, followed by two days without injections. This treatment cycle was repeated 3 times, lasting 21 days. On day 23, animals were anesthetized with a ketamine (20 mg/ml)/xylazine (2mg/ml) cocktail (5μl/g) and received intracardiac injections of established C4-2 PCa cell lines (250,000 in 20 μl). Groups 1 and 5 received WT cells, Groups 2 and 6 received control shRNA cells, encoding a non-specific scrambled shRNA sequence, Groups 3 and 7 received TRP-11 cell line, which had a decrease in TRPV6 expression of ~60%, and Groups 4 and 8 received TRP-12 cell line, which had a decrease in TRPV6 expression of ~63%. After PCa cell inoculation, animals received an additional 5 days of intermittent PTH 1-34 or vehicle injections. Eight weeks post-PCa cell injection mice were administered ketamine/xylazine cocktail and blood was collected via cardiac puncture before euthanasia via cervical dislocation. Gross necropsies were performed, followed by the extraction of the heart, lungs, spleen, liver, hind limbs and lumbar vertebrae. Soft tissues were weighed, fixed overnight in 4% formaldehyde and stored at 4°C in 70% ethanol. Hindlimbs and lumbar vertebrae were cleaned of all soft tissue, and fixed for 24-48 hrs in 4% formaldehyde, then subsequently decalcified for 14-days in 10% EDTA, pH 8 (4°C) and stored at 4°C in 70% ethanol until paraffin embedding. This experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Pennsylvania State University College of Medicine [74, 85].
4.3.5 Prostate Specific Antigen ELISA

Blood samples were kept at 4°C overnight to clot then centrifuged at 10,000 G for 10 min to separate out serum. Serum was analyzed for the presence of PSA using an ELISA according to the manufacturer’s protocol (Anogen, Mississauga, Ontario, Canada). PSA serum amounts were calculated from a standard curve created from known concentrations of PSA. Animals were considered PSA positive if serum levels exceeded 4.5 ng/ml [74, 85].

4.3.6 Immunohistochemistry

Localization of pan-cytokeratin and PSA positive cells within harvested tissues were performed by indirect immunohistochemistry employing polyclonal antibodies. Sections were deparaffinized and rehydrated by passage through xylene and graded alcohol series to water, then blocked using 3% (v/v) normal goat serum and incubated overnight (4°C) with primary antibodies specific to PSA and pan-cytokeratin. Bound antibodies were visualized using ImmPACT DAB Peroxidase substrate (Vector Labs, Burlingame, CA) as a chromagen. Sections were counter-stained with either Hematoxylin or Methyl Green. Antibody specificity was assessed by staining normal mouse lung tissue and incubating tissue sections with only secondary antibodies [74, 85].

In order to determine if there was a statistically significant difference in the number of PSA positive cells in animals from different treatment groups, PSA positive cells were counted in the marrow spaces of the proximal femurs, distal femurs, and proximal tibiae. Three 20X images were captured per animal using a Nikon EFD3 inverted light microscope and PSA positive cells per µm² were quantified using NIS Elements-D 3.2 software (Melville, NY).
4.3.7 Statistical Analysis

All experiments were conducted in at least triplicate and repeated at least three times. Graphpad PRISM version 5.0 (GraphPad Software, San Diego CA) was used for statistical analysis. One-way ANOVA with a Bonferroni post hoc test was performed with experiments of multiple groups to compare all columns of data. Fisher’s exact test was used to determine statistical significance of PSA and pan-cytokeratin positive animals. All data are expressed as mean ± SD and were considered significant if p ≤ 0.05.

4.4 Results

4.4.1 End Stage Body and Tissue Weights

Throughout the experimental time course animals gained an average of 3.05 g (26.4 ± 0.85 g mean initial and 29.4 ± 1.145 g mean final body weight). At no time during the study were differences in mean body weight observed between treatment groups (Figure 4.1). Some mice did not survive the duration of the study, two mice died the day of PCa cell inoculations. A week after PCa cell inoculation, 3 mice died all animals had received PTH treatment, 2 were injected with WT cells while the other was injected with control shRNA cells.

Wet weights of lung, liver, heart, and spleen were measured and there were no differences in mean weight of any of the organs between the different treatment groups (Figure 4.2). Two mice developed lung tumors which increased their lung weight and were considered outliers (Figure 4.2A). One animal with a lung tumor (WT/PTH treatment group) also had increased spleen (Figure 4.2C) and liver wet weight (Figure 4.2D).
4.4.2 Prostate Specific Antigen Serum Analysis

Serum PSA analysis identified two mice expressing PSA above the 4.5 ng/ml threshold. One mouse also had a prominent lung tumor. All other animals had PSA levels that ranged from 2.1-2.8 ng/ml and were considered PSA negative.

4.4.3 Immunohistochemistry

Lung tissue sections from the animals that developed lung tumors were probed with antibodies specific to PSA. While the metastatic lesions encompassed approximately 90% of the lung tissue, PSA positive cells were only localized along the periphery of the tumor near vascular elements. Lung tumor tissue sections exhibited a dense cellular morphology and very few alveoli and bronchi were present. In contrast, tumor negative lung tissues were also negative for PSA positive cells (Figure 4.4).

Serial sections of femurs, proximal tibiae, and lumbar vertebrae were probed with antibodies specific to PSA and pan-cytokeratin, markers for PCa epithelial cells. PCa cells were identified in small clusters (3-5 cells) within the marrow spaces of bone tissue sections in close proximity to growth plates and vascular elements. In regard to long bones, 100% of animals administered PTH and injected with WT C4-2 cells had PSA and pan-cytokeratin positive cells visualized in the marrow spaces of the distal femur and proximal tibia. In contrast, 20% of animals administered vehicle and injected with WT C4-2 cells had PSA and pan-cytokeratin positive cells visualized within the marrow spaces of long bones. These results are consistent with our previous report [74].

PSA and pan-cytokeratin positive cells were visualized in 60% of the animals injected with control shRNA cells and administered PTH while 50% of animals administered vehicle had PSA and pan-cytokeratin positive cells which was not statistically different. In contrast,
only 20% of animals injected with TRP-11 cells and administered PTH had PSA and pan-cytokeratin positive cells observed in the long bones; this difference was statistically significant. However, TRP-12 colonization of bone was similar to animals injected with control shRNA cells (60%) (Table 4.1). PSA positive cells were quantified by individual region (proximal femur, distal femur, and proximal tibia) to determine if there was a decrease in cell number when cells with reduced TRPV6 expression colonized bone. There was no difference observed (data not shown); there were also no differences observed when all three regions were combined (Figure 4.6).

In comparison to long bones, there were disparities in our observations of lumbar vertebrae. PSA and pan-cytokeratin positive cells were visualized in the marrow spaces of the lumbar vertebrae in 67% of animals administered PTH and injected with WT C4-2 cells. Conversely, 40% of animals administered vehicle and injected with WT C4-2 cells had PSA and pan-cytokeratin positive cells visualized within the marrow spaces of lumbar vertebrae. PSA and pan-cytokeratin positive cells were visualized in 100% of the animals injected with control shRNA cells and administered PTH while 58% of animals administered vehicle had PSA and pan-cytokeratin positive cells identified in the lumbar vertebrae. In contrast, only 40% of animals injected with TRP-11 and TRP-12 cells and administered PTH had PSA and pan-cytokeratin positive cells observed in the lumbar vertebrae (Table 4.2). There was no difference in the number of PSA and pan-cytokeratin positive animals between groups.
<table>
<thead>
<tr>
<th>Group</th>
<th>PSA + Long Bones</th>
<th>Cytokeratin + Long Bones</th>
<th>Average % of PCa Positive Long Bones</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/Veh</td>
<td>1/5</td>
<td>1/5</td>
<td>20%*</td>
</tr>
<tr>
<td>WT/PTH</td>
<td>6/6</td>
<td>6/6</td>
<td>100%</td>
</tr>
<tr>
<td>Con/Veh</td>
<td>3/6</td>
<td>3/6</td>
<td>50%</td>
</tr>
<tr>
<td>Con/PTH</td>
<td>3/5</td>
<td>3/5</td>
<td>60%</td>
</tr>
<tr>
<td>TRP-11/Veh</td>
<td>2/5</td>
<td>2/5</td>
<td>40%</td>
</tr>
<tr>
<td>TRP-11/PTH</td>
<td>1/5</td>
<td>1/5</td>
<td>20%*</td>
</tr>
<tr>
<td>TRP-12/Veh</td>
<td>5/6</td>
<td>6/6</td>
<td>92%</td>
</tr>
<tr>
<td>TRP-12/PTH</td>
<td>3/5</td>
<td>3/5</td>
<td>60%</td>
</tr>
</tbody>
</table>

Table 4.1 Number of animals per group with PSA and pan-cytokeratin positive cells present in marrow spaces of the femur and proximal tibia (Long bones). The right most column gives the average percentage of animals per group that were both PSA and pan-cytokeratin positive. * indicates significantly different compared to the WT/PTH group, p ≤ 0.05.
<table>
<thead>
<tr>
<th>Group</th>
<th>PSA+ Lumbar</th>
<th>Cytokeratin + Lumbar</th>
<th>Average % of PCa Positive Lumbar</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/Veh</td>
<td>2/5</td>
<td>2/5</td>
<td>40%</td>
</tr>
<tr>
<td>WT/PTH</td>
<td>4/6</td>
<td>4/6</td>
<td>67%</td>
</tr>
<tr>
<td>Con/Veh</td>
<td>5/6</td>
<td>2/6</td>
<td>58%</td>
</tr>
<tr>
<td>Con/PTH</td>
<td>5/5</td>
<td>5/5</td>
<td>100%</td>
</tr>
<tr>
<td>TRP-11/Veh</td>
<td>2/5</td>
<td>3/5</td>
<td>50%</td>
</tr>
<tr>
<td>TRP-11/PTH</td>
<td>2/5</td>
<td>2/5</td>
<td>40%</td>
</tr>
<tr>
<td>TRP-12/Veh</td>
<td>3/6</td>
<td>6/6</td>
<td>75%</td>
</tr>
<tr>
<td>TRP-12/PTH</td>
<td>2/5</td>
<td>2/5</td>
<td>40%</td>
</tr>
</tbody>
</table>

Table 4.2 Number of animals per group with PSA and pan-cytokeratin positive cells present in marrow spaces of the lumbar vertebrae. The right most column gives the average percentage of animals per group that were both PSA and pan-cytokeratin positive.
Figure 4.1 Terminal body weights did not differ among treatment groups. Animal weights were taken on the final day of the study. No differences were observed in animal weights between groups. The bars represent the mean weight (g) +/- SD per group. n=5-6/group, V= vehicle, P= PTH
Figure 4.2 Scatter plots of soft tissue wet weights. A) Lung tumors were visualized in two animals, indicated by orange color. B) Heart. C) Spleen. One animal with a prominent lung tumor also had an enlarged spleen (indicated in orange). D) Liver. No differences in wet weights of soft tissues were observed. \( p \leq 0.05 \), \( n=5-6/\)group, \( V = \) vehicle, \( P = \) PTH
Figure 4.3 Scatter plot of PSA serum levels across treatment groups. Serum collected 8 weeks after PCa cell inoculation was analyzed by ELISA for PSA. Two animals were 4.5 ng/ml or above, suggesting they were PSA positive (indicated by the red circle and orange square). For the other animals, serum PSA levels ranged from 2.1-2.8 ng/ml and were considered PSA negative. Another animal with an observed lung tumor was not PSA positive (indicated by the black outlined orange square). There were no significant differences in serum PSA among groups. $p \leq 0.05$, n=5-6/group, V= vehicle, P= PTH
Figure 4.4 PSA positive cells were localized within lung tumors. Paraformaldehyde fixed lung tissue was stained with hematoxylin & eosin (H&E) and probed with antibodies specific for PSA. 20X images representing normal tissue (top row) and tumorous tissue (bottom row). Positive staining is indicated by the yellow arrows. Scale bar = 0.1 mm (black bar bottom right image)
Figure 4.5 PSA and pan-cytokeratin cell localization in long bones of mice inoculated with wild type cells. Long bone tissue sections were deparaffinized and rehydrated by passage through xylene and graded alcohol series to water, then blocked using 3% (v/v) normal goat serum and incubated overnight (4°C) with primary antibodies specific to PSA and pan-cytokeratin or stained with H&E. Bound antibodies were visualized using ImmPACT DAB Peroxidase substrate as a chromagen. Sections were counter-stained with either Hematoxylin or Methyl Green. Positive cells are marked by the yellow arrows indicating brown pericellular staining. The labels on the left indicate administration of PTH or vehicle (Veh). Scale bar = 0.1 mm (bottom right image)
Figure 4.6 PSA and pan-cytokeratin cell localization in long bones of mice inoculated with control shRNA cells. Long bone tissue sections were deparaffinized and rehydrated by passage through xylene and graded alcohol series to water, then blocked using 3% (v/v) normal goat serum and incubated overnight (4°C) with primary antibodies specific to PSA and pan-cytokeratin or stained with H&E. Bound antibodies were visualized using ImmPACT DAB Peroxidase substrate as a chromagen. Sections were counter-stained with either Hematoxylin or Methyl Green. Positive cells are marked by the yellow arrows indicating brown pericellular staining. The labels on the left indicate administration of PTH or vehicle (Veh). Scale bar = 0.1 mm (bottom right image)
Figure 4.7 PSA and pan-cytokeratin cell localization in long bones of mice inoculated with TRP-11 cells. Long bone tissue sections were deparaffinized and rehydrated by passage through xylene and graded alcohol series to water, then blocked using 3% (v/v) normal goat serum and incubated overnight (4°C) with primary antibodies specific to PSA and pan-cytokeratin or stained with H&E. Bound antibodies were visualized using ImmPACT DAB Peroxidase substrate as a chromagen. Sections were counter-stained with either Hematoxylin or Methyl Green. Positive cells are marked by the yellow arrows indicating brown pericellular staining. The labels on the left indicate administration of PTH or vehicle (Veh). Scale bar = 0.1 mm (bottom right image)
Figure 4.8 PSA and pan-cytokeratin cell localization in long bones of mice inoculated with TRP-12 cells. Long bone tissue sections were deparaffinized and rehydrated by passage through xylene and graded alcohol series to water, then blocked using 3% (v/v) normal goat serum and incubated overnight (4°C) with primary antibodies specific to PSA and pan-cytokeratin or stained with H&E. Bound antibodies were visualized using ImmPACT DAB Peroxidase substrate as a chromagen. Sections were counter-stained with either Hematoxylin or Methyl Green. Positive cells are marked by the yellow arrows indicating brown pericellular staining. The labels on the left indicate administration of PTH or vehicle (Veh). Scale bar = 0.1 mm (bottom right image)
Figure 4.9 Number of PSA positive cells quantified in long bones did not differ among treatment groups. Images were taken of the proximal femur, distal femur, and proximal tibia of tissue sections probed with antibodies specific to PSA. PSA positive cells were quantified across three images from each region for each animal. Each bar represents the mean number +/- SD of PSA positive cells per µm² per treatment group.
4.5 Discussion

TRPV6 ion channels have previously been linked to PCa tumor progression and are thought to promote PCa cell growth and survival [107, 127]. Our preliminary studies in vitro suggested that cells with decreased TRPV6 expression had slower rates of proliferation, decreased heterotypic cell adhesion, diminished ability to migrate and to invade hbmE cell monolayers. We aimed to determine the role of TRPV6 ion channels in the early colonization of osteoblastic PCa cells of murine bone. To test this hypothesis C4-2 cells expressing basal and low levels of TRPV6 were injected into SCID/Beige mice after administration of intermittent PTH 1-34. Immunohistochemical analysis of skeletal sites suggested that WT cells colonized 100% of the long bones of animals administered PTH, which was consistent with our previous observations [74]. In contrast, TRP-11, one of the clones with stably reduced levels of TRPV6 expression, colonized the long bones of only one animal administered PTH. However, TRP-12 colonized 60% of the long bone of animals administered PTH. These observations, while preliminary, suggest that TRPV6 may have a role in the early colonization of osteoblastic PCa cells to murine bone.

Five mice died before the end of the study. Two of these mice died following inoculation with PCa cells, which may have been caused by the intracardiac injection or a complication of anesthesia. Two animals administered PTH and injected with WT cells died as a result of the aggressiveness of their cage mates. The last animal was administered PTH and injected with control shRNA cells, the cause of death was not determined. There were 6 animals per group at the beginning of the study, but because of the unexpected deaths the power of this study was reduced from 95% to 90%. Fortunately we were still able to detect
significant differences between some groups; however, it is difficult to determine how our results would differ with a larger sample size.

Two animals developed prominent lung tumors eight weeks following PCa cell injection. PCa cells have been reported to spontaneously metastasize to the lungs, albeit at a lower percentage than to bone [188, 189]. Cell lines from the LNCaP lineage have been reported to form lung metastases [190]. In contrast to our study, Fu and colleagues injected C4-2B PCa cells into the prostate gland of C.B. 17 mice and observed prominent lung tumors 10 weeks following injection. Our results are also consistent with the human disease, PCa metastases were analyzed from approximately 1600 men and lung metastases were found in 46% of men while 90% of men had established bone metastases [189]. These animals received different cells lines and it is possible that during intracardiac injections the heart was missed and the cells were injected into the lungs. The vast majority of the tumors were not PSA positive, confirmed by immunohistochemistry, however, there were PSA positive cells localized along the periphery of the tumors near vascular elements. While every cell line expressed PSA, as confirmed by Western blot analysis prior to injection (data not shown), the cells may have undergone epithelial to mesenchymal transition in vivo which led to their decrease in PSA secretion [64, 191].

PSA serum analysis has traditionally been used as a marker for PCa [192]. We found that only two animals injected with PCa cells had elevated serum levels of PSA suggesting that viable PCa cells were present. All animals had detectable low levels of serum PSA (2.1-2.8 ng/ml) which were below the PSA positive range of 4.5 ng/ml and it is possible that in vivo PCa cells produced lower levels of PSA. Thalmann et al. reported animals that developed osseous metastases from C4-2 cells had reduced or lacking serum PSA levels
To determine if PSA positive PCa cells were within the circulation, qPCR could have been conducted with RNA extracted from blood samples. Regardless, previous studies employing C4-2 cells in a murine animal model, have reported the development of lymph node and bone metastases with undetectable levels of serum PSA [64]. Further, in vivo studies with C4-2 sub-lines have reported that PSA serum levels decline over time and animals that developed metastatic lesions tended to secrete lower amounts of PSA [69]. These observations suggest that perhaps C4-2 cells have an adaptive response to the bone microenvironment which results in a down regulation of PSA secretion. This is supported by the results of the immunohistochemistry suggesting that PSA positive cells were present in the long bones and lumbar vertebrae of animals considered negative for serum PSA.

Immunohistochemical analysis revealed WT C4-2 PCa cells in the long bones and lumbar vertebrae localized near growth plates and vascular elements. These results were consistent with our previous studies [74]. While our lab, employing this model, has not reported PSA positive cells localized in the lumbar vertebrae until now, C4-2 cells have been reported to develop metastatic lesions in the spine of athymic and SCID/Beige mice [64, 193]. Cells from the TRP-11 clone were identified in a smaller percentage of long bones compared to lumbar vertebrae. The differences observed between the lumbar vertebrae and long bones could have been a result of variations in load and/or bone turnover [194]. Previously, we have reported that PTH 1-34 has divergent effects depending on location (i.e.- femur, tibia, lumbar vertebrae). MicroCT analysis of bones from animals administered PTH 1-34 detected an increase in bone mineral density in the distal femur but not the lumbar vertebrae [85].
We speculate that with a larger sample size the differences we observed in the long bones would be consistent with the lumbar vertebrae. A power analysis revealed that in order to detect a significant proportional difference between animals that are positive or negative for PCa cells, a sample size of at least 12 animals would be needed for a power of 95% (Graphpad Statmate, San Diego, CA). A power analysis of our previous studies suggested to attain a power of 95% we would have needed 5 animals; 6 animals should have been sufficient for this study. While we have not experienced the loss of animals due to anesthesia complications and litter mate aggressiveness in the past, we could have better prepared for unexpected complications and increased the number of animals.

PSA and pan-cytokeratin positive cells were identified in 100% of the long bones of animals administered PTH 1-34 and injected with WT cells. In comparison, PSA and pan-cytokeratin positive cells were identified in only 20% of the long bones of animals administered vehicle and injected with WT. These observations are consistent with the limited studies employing PTH 1-34 to enhance the uptake of PCa cell to murine bone [53, 74]. In contrast, administration of PTH 1-34 did not increase the percentage of long bones with PSA and pan-cytokeratin positive cells in animals injected with control shRNA cells. Off target effects from the scrambled shRNA may explain the disparities in our observations [174]; however, our in vitro studies suggested that the control shRNA cells were similar to the WT cell line in regards to proliferation, adhesion, migration, and invasion. Moreover, off target effects are less common with shRNA applications and have been reported to be dose dependent [174], within our study we used only one infectious unit/cell which was considerably lower than the suggested dose of ten infectious units/cell (Santa Cruz).
The percentage of positive cells localized in animals injected with TRP-12 did not differ from animals injected with WT cells; it is possible that without selective pressure in vivo, the cells expressed normal levels of TRPV6 [170]. In order to confirm this possibility, we could have used green fluorescent protein (GFP) lentiviral particles to monitor the presence of shRNA constructs within the cells [195]. This approach is commonly used to ensure the presence of shRNA constructs as well as monitor cancer cells in vivo [196]. We could have also added doxycycline hydrochloride to the animals’ drinking water to ensure injected cells were still under selective pressure [170]. Our in vitro studies suggested that TRP-12 cells had reduced migration, while there were no differences observed with TRP-11 cells which suggest these cell lines were different. These in vitro observations suggested that TRP-12 might not colonize bone as well as TRP-11 and provide support that in vivo TRP-12 may have expressed basal levels of TRPV6. It is interesting that only two animals administered PTH and injected with TRP-11 had PSA and pan-cytokeratin positive cells identified in the long bones and lumbar vertebrae, this observation suggests a role for TRPV6 in the early colonization of C4-2 cells to murine bone.

The number of PSA and pan-cytokeratin positive cells was not different among treatment groups. This observation suggested that when cells with reduced levels of TRPV6 were able to reach the bone environment they acted similar to WT cells. It is possible that because of the small area sampled, ~150 microns of bone, tissue sections were under sampled, nonetheless, we took every effort to ensure that a representative sample of bone from each specimen was examined as we and others have done previously [69, 74, 85]. Another alternative to quantifying micrometastases would be to extend the study until
osseous lesions were visible via bone scans, however, this approach would not directly examine the early uptake and colonization of PCa cells to murine bone.

In our previous study, we assessed PCa cell viability and colonization by measuring serum PSA. 70% of the animals were PSA positive 8 weeks post inoculation [74]. We used the 8 week time point based on our previous study but perhaps extending the study would have been beneficial. Murine models employing PC-3 PCa cells have been reported to develop primarily osteolytic micrometastases in the long bones and craniofacial regions of mice 5 weeks following intracardiac injection although these cells are more aggressive than C4-2 cells [52, 53]. It has been reported that C4-2 cells sporadically develop bone metastases 16-24 weeks post injection [3]. Other studies reported animals that received intracardiac or subcutaneous injections of C4-2 cells developed metastatic lesions within 11-29 weeks, although at a lower incidence rate (24%) than we have observed [64, 69, 193, 197]. Considering these studies, our future experiments could extend the period between PCa cell injection and end stage analysis to at least 10 weeks to allow for a longer period for micrometastases to develop.

We were the first to use osteoblastic PCa cell lines with decreased TRPV6 expression in vivo. Our results suggested that a reduction in TRPV6 ion channel expression, in at least one clone, significantly decreased PCa cell colonization to murine long bones. Our data contributes to the existing literature, which suggests TRPV6 ion channel expression correlates with PCa aggressiveness [107, 126] and that TRPV6 is necessary for PCa cell proliferation and survival, in vitro [127]; our observations though largely preliminary suggest TRPV6 ion channels may have a role in the uptake and colonization of PCa cells in bone. Although, future studies are warranted to confirm these observations, studies targeting
TRPV6 should be continued to eventually develop therapeutic agents that prevent the development of PCa bone metastases.
Chapter 5:

Summary of this Dissertation and Future Directions
5.1 Summary of this Work

The studies presented herein were conducted to identify the effect of transient increases in extracellular calcium on the metastatic potential of osteoblastic PCa cells and to elucidate the role that TRPV6 ion channels have in this process. This work was predicated on the observation that administration of intermittent PTH enhanced the uptake and colonization of osteoblastic PCa cells to murine bone. Additionally, in response to PTH, serum calcium levels transiently increase which we propose confers a metastatic advantage to PCa cells within circulation. The metastatic cascade includes a series of processes that enable a cancerous cell to exit the primary tumor and colonize a distant target. We analyzed independent but related components of the metastatic cascade including proliferation, survival, adhesion, and migration, *in vitro*.

Our observations suggested that transient elevations in extracellular calcium impact PCa metastatic potential in disparate ways. We observed no differences in PCa cell proliferation or viability when exposed to transient increases in extracellular calcium. Interestingly, our data suggested that osteoblastic PCa cell adhesion to hbmE cell monolayers was significantly enhanced when hbmE cells were exposed to CaCl$_2$. It is well documented that PCa cells preferentially bind to bone marrow endothelial cells relative to other endothelial cells [134, 198]. The studies herein provide further evidence that *in vivo*, PCa cells within circulation may bind to bone marrow endothelial cells exposed to high concentrations of calcium at an increased incidence.

We further provided evidence to suggest that this increase in adhesion was due to an increase in E-Selectin, VCAM-1, and ICAM-1 cell surface abundance. It has been suggested that E-Selectin, ICAM-1, and VCAM-1 are involved in PCa progression; several groups have
observed an increase in these molecules within the serum of patients with advanced metastatic PCa [163-166]. Further, E-Selectin and VCAM-1 have been reported to be necessary for the adhesion of hematopoietic progenitor cells to bone marrow endothelial cells and subsequent migration to reach the bone marrow compartment [140]. Our studies suggest that E-Selectin and VCAM-1 may also have a role in arresting osteoblastic PCa cells within circulation because when their expression was blocked by function blocking antibodies, adhesion to C4-2 cells was decreased by 50%. It is well understood that TNFα and IL-1 can activate E-Selectin, ICAM-1, and VCAM-1 which increases their cell surface abundance within 4-6 hrs [142]. Our observations suggest that extracellular calcium can also increase E-Selectin, ICAM-1, and VCAM-1 cell surface abundance within 6 hrs. Previous studies have reported increases in E-Selectin and VCAM-1 in response to an increase in intracellular calcium [143], but our data suggests that extracellular calcium may independently be involved in the cell surface abundance of these molecules. However, we propose that other adhesion molecules are involved in this process and further studies are necessary to identify these molecules and to fully understand the interaction between bone marrow endothelial cells and osteoblastic PCa cells.

We also identified that extracellular calcium could act as a chemoattractant to enhance osteoblastic PCa cell migration and invasion through hbmE cell monolayers. There have been few studies to specifically study the role of extracellular calcium in PCa cell migration and invasion; however, the CaSR has recently emerged as a mediator of bone metastases. Observations suggest the CaSR is required for the migration of metastatic cancer cells [52, 128, 146]. These studies are consistent with our observations which suggested the CaSR expression in osteoblastic PCa cells was enhanced following exposure to elevated
extracellular calcium. We also provide evidence, for the first time, that extracellular calcium enhances TRPV6 expression; it is possible that a combination of the CaSR and TRPV6 may have a role in the progression of PCa. Our in vitro observations suggest that increases in serum calcium may serve to alter the adhesive properties of hbmE cells which allow PCa cells to better adhere to these cells and increase directional migration of PCa cells into the bone microenvironment.

Due to the disparate severity in PCa treatment, there is a need for markers which assist in the identification of aggressive PCa [28]. One potential marker is TRPV6, which is not present in healthy prostate tissue. Its expression, however, increases as localized PCa becomes more aggressive [107, 126]. TRPV6 expression was enhanced in response to transient increases in extracellular calcium which suggest that in vivo, PTH 1-34 administration may indirectly, through transient spikes in serum calcium levels, increase TRPV6 expression in PCa cells within circulation.

This observation led us to stably decrease TRPV6 expression in C4-2 PCa cell lines using shRNA lentiviral particles. We determined that osteoblastic PCa cells with decreased TRPV6 expression proliferate at a slower rate and have decreased adhesive and invasive properties compared to WT and control shRNA cells. We observed a decrease in adhesion in cells with reduced TRPV6 expression and demonstrated that these cells poorly adhered to hbmE cell monolayers exposed to CaCl₂ which enhanced the adhesion of WT C4-2 cells. The Lehen’kyi group is the only other group that has published regarding TRPV6 and PCa. They observed a decrease in LNCaP cell proliferation when TRPV6 expression was reduced [127]. Our observations were consistent with this group but we also provided evidence to suggest that TRPV6 has a role in adhesion and invasion of osteoblastic PCa cells. Further, other than
an observation that TRPV6 expression was increased in endometrium during implantation [177], there was little evidence to suggest an adhesive role for TRPV6 prior to the completion of this work.

These observations suggested that TRPV6 expression was related to adhesion molecules present on osteoblastic PCa cells. One potential molecule could be CD44 which is a ligand for E-Selectin [178] and was decreased in cells with reduced TRPV6 expression. Further investigation is warranted to link CD44 to TRPV6; however, one possible way in which decreased TRPV6 expression could result in decreased CD44 expression is through the S100A10-annexin 2 complex.

Our data suggested that TRPV6 had a role in adhesion and invasion of osteoblastic PCa cells. These cellular processes could be required for the development of PCa bone metastases; therefore, we asked whether cells with decreased TRPV6 expression could still develop micrometastases in murine bone. SCID/Beige mice were administered intermittent PTH 1-34 and injected with C4-2 cells expressing basal and low levels of TRPV6. PSA and pan-cytokeratin positive cells were present in 100% of the long bones of animals injected with WT cells, which is consistent with our previous observations [74]. In contrast, we observed PSA and pan-cytokeratin positive cells in long bone tissue sections of only one animal injected with the TRP-11 clone with stably reduced levels of TRPV6 expression. However, we observed PSA and pan-cytokeratin positive cells in 60% of animals administered PTH and injected with the TRP-12 clone. These observations suggested that at least a clone with decreased TRPV6 expression poorly colonized murine long bones.

While preliminary, this is the first in vivo study to suggest a role for TRPV6 in the uptake and colonization of osteoblastic PCa cells to murine bone. Potential limitations of this
study include the small number of animals used to complete the study which was due to unexpected complications. Additionally, there were disparities between our in vitro and in vivo studies with regard to control shRNA cell lines and TRP-12 cell lines. Control shRNA cell lines acted similarly to WT cell lines in vitro, however, colonized bone at a lower incidence. PTH 1-34 administration did not appear to have an effect on these cells which may suggest off target effects from the scrambled control shRNA lentiviral particles [174]. There was also a concern that TRP-12 cell lines re-established basal levels of TRPV6 expression once in vivo because they were not kept under selective pressure which may explain why they were visualized in a similar number of long bones as animals injected with WT cell lines.

The National Cancer Institute estimates that every year approximately 230,000 men are diagnosed with PCa and 30,000 are expected to succumb to the disease in the United States. About 60-85% of men with advanced PCa have associated bone metastases [6]. PCa bone metastases contribute to bone pain and an increased risk of fracture which can potentially lead to paralysis due to spinal cord compression [7]. There are currently no effective therapeutic treatments available to prevent or fully eradicate PCa bone metastases [1, 8]. Our goal was to elucidate the cellular mechanisms involved in the early colonization of PCa cells to bone. Toward this goal, we have attained a better understanding of the role of extracellular calcium and TRPV6 in osteoblastic PCa cells. The current study is the first to demonstrate that transient increases in extracellular calcium enhance adhesion and migration of osteoblastic PCa cells and that this enhancement may be due to TRPV6 expression. Our observations suggest that TRPV6 ion channels modulate osteoblastic PCa cell proliferation, adhesion, and invasion. Further, we provide evidence to suggest that TRPV6 ion channel
expression is linked to CD44, a ligand for E-selectin. Our preliminary animal study also provided evidence to suggest that TRPV6 ion channels may have a role in the early colonization of PCa cells to murine bone. This thesis provides further evidence to suggest that TRPV6 ion channel expression is correlated with PCa progression. Although these observations together support our hypothesis, future studies are warranted to identify the specific cellular mechanisms responsible.

5.2 Future Directions

5.2.1 The interaction between PCa cells and Bone Marrow Endothelial Cells

Future studies should focus on the interaction between PCa cells and hbmE cells. A better understanding of this interaction could lead to more effective treatments to prevent the development of PCa bone metastases. Our studies suggest that increases in extracellular calcium enhance the adhesion and invasion of PCa cells to hbmE cell monolayers. We further provided evidence that suggested in response to elevations in extracellular calcium, E-Selectin, ICAM-1, and VCAM-1 increased cell surface abundance. It is likely that other adhesion molecules are involved in this process such as integrins and immunoglobulins [139]. Future studies should aim to identify these molecules using similar methods employed in this thesis to lead to the eventual advancement of therapeutic agents to prevent the development of PCa bone metastases.

Further, adhesion molecule ligands present on osteoblastic PCa cells need to be identified. We identified that CD44 expression was decreased in cells with decreased TRPV6 expression. This observation needs further investigation to determine the exact molecular interaction between TRPV6 and CD44. Immunocytochemistry could confirm co-localization of TRPV6 and CD44. Pull down assays and co-immunoprecipitations could help establish a
physical interaction between CD44 and TRPV6. We suggested that TRPV6 is able to influence CD44 expression via the S100A10-Annexin-2 complex. Others have reported that S100A10 interacts with the C-terminal tail of TRPV6 [116], this interaction could be confirmed in C4-2 PCa cell lines employing a yeast two hybrid system using the full length S100A10 coding sequence and the C-terminal tail of TRPV6. Glutathione S-transferase (GST) pull down assays with GST-TRPV6 fusion proteins immobilized on Glutathione Sepharose beads could be performed with S100A10, Annexin-2, and CD44. To establish that CD44 is responsible for the observed decrease in adhesion to hbmE cells, function blocking antibodies and siRNA specific to CD44 could be utilized to decrease CD44 expression in C4-2 cells and adhesion assays could be repeated. These experiments would lead to a better understanding of the interactions between osteoblastic PCa cells and bone marrow endothelial cells.

5.2.2 TRPV6 Signaling Pathway

To determine if the CaSR is necessary for TRPV6 function and/or expression, siRNA specific to the CaSR could be used and TRPV6 expression would be measured. Our observations suggested that TRPV6 and CaSR protein expression was enhanced in response to elevated extracellular calcium. If the CaSR was necessary for the increase in TRPV6 protein expression, then cells treated with siRNA-CaSR would not respond to changes in extracellular calcium. To determine the cellular signaling pathway between the CaSR and TRPV6, signaling intermediates could be blocked with siRNA including phospholipase C, PI3K, PIP2, and PKC and similar experiments could be employed to determine if their expression was necessary for TRPV6 expression.
5.2.3 Future *in vivo* studies

Future *in vivo* studies employing cells with decreased TRPV6 are warranted. If we were to repeat our *in vivo* study, we would employ fluorescently labeled C4-2 cells with reduced TRPV6 expression to facilitate *in vivo* imaging to track the localization of PCa cells after injection and quantify the number of cells that colonize the bone environment [53]. Furthermore, the experiment would be extended until animals injected with WT C4-2 cells developed visually apparent metastatic lesions identified by bone scans to provide insight into the role of TRPV6 ion channels in the establishment of metastatic lesions. Alternatively, to determine whether TRPV6 ion channels are necessary for establishment of PCa cells in the bone microenvironment, PCa cells with reduced levels of TRPV6 expression could be directly injected into tibiae of SCID/Beige mice and tumor burden could be evaluated by PSA serum analysis and bone scans over a period of at least 15 weeks [57]. To test the hypothesis that TRPV6 ion channels are necessary for tumor growth, cells could be injected subcutaneously into athymic mice and tumor size could be measured over time. These studies, if conducted, would provide further insight to the role of TRPV6 ion channels in PCa progression.

5.2.4 Over-expression of TRPV6

To fully understand the role of TRPV6 in metastatic PCa cells, TRPV6 should be over-expressed in osteoblastic PCa, non-metastatic PCa, and normal prostate epithelial cell lines. Full length cDNA clones for TRPV6 would be sub-cloned into a pcDNA3.1 protein expression vector. Over expression would be confirmed by qPCR and Western blot analysis and control cells would be cloned with only the pcDNA3.1 vector. The cells would be characterized *in vitro* as previously done to ascertain changes in proliferation, adhesion, and
invasion and then used for \textit{in vivo} experiments to determine if increased TRPV6 expression increases the aggressiveness of PCa cells. We would expect that the metastatic potential of cells with increased TRPV6 expression would have enhanced proliferative, adhesive, and invasive properties and would form metastatic lesions more readily than WT C4-2 cells.
Appendix A:

Short-term Intermittent PTH 1-34 Administration Enhances Bone Formation in SCID/Beige Mice

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A.1 Abstract

The anabolic effect of intermittent PTH on bone is variable depending on the species studied, duration/mode of administration, and location of skeletal response investigated. We tested the hypothesis low dose, short term, intermittent PTH 1-34 administration is sufficient to enhance bone formation without altering bone resorption. To test our hypothesis, mice were treated intermittently with one of three concentrations of PTH 1-34 (1 μg/kg; low, 10 μg/kg, or 20 μg/kg; high) for three weeks. The skeletal response was identified by quantifying: serum markers of bone turnover, cancellous bone parameters in distal femur, proximal tibia, and lumbar vertebrae by µCT, and number of osteoblasts and osteoclasts in distal femur. Mice receiving 20 μg/kg of PTH 1-34 demonstrated a 30% increase in serum osteocalcin, but no differences in serum calcium, type I collagen teleopeptides, or TRACP 5b. For all bones, µCT analysis suggested mice receiving 20 μg/kg of PTH 1-34 had increased cancellous bone mineral density, trabecular thickness and spacing, but decreased trabecular number. A 60% increase in the number of alkaline phosphatase positive osteoblasts in the distal femur was also observed in tissue sections; however, the number of TRAP positive osteoclasts was not different between test and control groups. While animals administered 10 μg/kg demonstrated similar trends for all bone turnover indices, such alterations were not observed in animals administered PTH 1-34 at 1 μg/kg per day. Thus, PTH 1-34, administered intermittently for three weeks at 20 μg/kg is sufficient to enhance bone formation without enhancing resorption.
A.2 Introduction

PTH is an 84 amino acid polypeptide secreted by parathyroid glands in response to low serum calcium. The exogenous administration of PTH can induce both anabolic and catabolic actions in bone; however, such consequences depend upon how PTH is administered. Continuous PTH infusion, similar to the condition of hyperparathyroidism, initiates bone catabolism by enhancing resorption and decreasing formation. In contrast, when administered intermittently (by daily injection), PTH promotes bone formation, as measured by increased bone mass and enhanced mechanical properties [78, 79, 199-201]. Interestingly, while PTH can be administered as a full length peptide, the first 34 amino acids (1-34) are sufficient for its biological activity in bone [76, 200, 202, 203]. Daily PTH 1-34 administration, similar to the full length peptide, is thought to increase bone formation primarily by enhancing the number of bone forming osteoblasts [83].

The anabolic action of intermittent PTH 1-34 on the skeleton has been widely studied in rodent models [80, 82, 204-209]. Studies employing murine models suggest the skeletal response to exogenous PTH 1-34 varies depending on the dose and duration of administration, as well as the mouse strain, age, and skeletal site investigated. Investigations of PTH 1-34 action on bone have employed doses ranging from 3-800 μg/kg of body weight [82, 84, 204-207, 209, 210] and durations lasting a couple of weeks to several months [77, 84, 206, 210-212]. In general, low dose (≤40 μg/kg), short term (≤ 3 weeks) intermittent PTH 1-34 administration enhances bone formation without altering bone resorption.

However, with administration of higher doses (≥ 40 μg/kg) and/or longer durations (≥ 4 weeks), bone turnover is enhanced by elevations in cellular activities supporting both formation and resorption [53, 77, 213]. Interestingly, not all skeletal sites respond equally to
PTH 1-34. In the majority of studies, intermittent PTH 1-34 administration consistently enhances trabecular bone formation in the femur; however, responses are more variable in the lumbar spine and tibia [80, 82, 84, 205]. Previous studies suggest, initial trabecular volume of skeletal sites and animal age are important considerations when investigating the bone anabolic effect of PTH in murine models [82, 84, 204, 205, 209, 214].

We recently employed intermittent PTH 1-34 administration to identify the role of bone formation in the colonization and establishment of human PCa in SCID/Beige bone [214]. Therein, we observed that short term intermittent PTH 1-34 administration (40 µg/kg) enhanced bone formative as well as resorptive indices (serum mTRACP 5b levels)[74]. We wondered if a lower dose of PTH 1-34 could enhance bone formation without elevating bone resorption. Identifying such a dose would be useful to future investigations seeking to define the role of bone formation in PCa colonization of bone. Unfortunately, the effects of low dose intermittent PTH 1-34 on bone turnover in SCID/Beige mice has not been reported. Thus, in this investigation, we tested the hypothesis that low dose, short term, intermittent PTH 1-34 administration to immune-compromised SCID/Beige mice is sufficient to enhance bone formation without altering bone resorption. To test our hypothesis, young-adult male mice were treated intermittently with one of three concentrations of human PTH 1-34 (1 µg/kg; low, 10 µg/kg, or 20 µg/kg; high), or vehicle, for three weeks. The skeletal response was evaluated by quantifying: serum levels of bone turnover markers (osteocalcin, mTRACP 5b, type I collagen teleopeptides, and calcium); changes in cancellous bone parameters in the distal femur, proximal tibia, and lumbar vertebrae by microcomputed tomography (µCT); and the number of active osteoblasts and osteoclasts in the distal femur.
A.3 Materials and Methods

Human parathyroid hormone fragment 1-34 (PTH 1-34) was purchased from Bachem Inc. (Torrance, CA). Enzyme-linked immunosorbent assay kits for serum osteocalcin, mouse specific tartrate resistant acid phosphatase 5b (TRACP-5b), and type I collagen teleopeptides were purchased from Biomedical Technologies Inc. (Stoughton, MA), Immunodiagnostics Systems Inc. (Fountain Hills, AZ), and Nordic Bioscience Diagnostics (Herlev, Denmark), respectively. The colorimetric assay, employed to quantify total serum calcium, was purchased from BioAssay Systems (Hayward, CA). Unless otherwise stated, all other reagents were purchased from Sigma Chemicals, Co (ST. Louis, MO).

A.3.1 Preparation of PTH

PTH 1-34 was reconstituted in 10 mM acetic acid/sterile phosphate buffered saline (PBS) solution and stored at -80°C at a concentration of 400 μg/ml in siliconized tubes (23). For injections, the stock solution was diluted to 50 μg/ml and then further diluted to 4 μg/ml, 2 μg/ml, and 1 μg/ml solutions for administration, control animals were injected with equivalent volumes of acetic acid diluted in PBS.

A.3.2 Animals

Thirty five, 5-6 week old, male, C.B-17 SCID/Beige mice were purchased from Taconic Laboratories (Rockville, MD), and stabilized at the animal research facility of the Penn State College of Medicine. Mice were housed five per cage, maintained under specific pathogen-free conditions, in a continuously HEPA filtered room, maintained between 21-22°C, with 40-60% humidity on 12 hr light and dark cycles and given free access to food and water. Animal body weights were recorded before the start and every other day during the experiment.
A.3.3 Experimental Design

At 7-8 weeks of age, mice were divided into four groups of equal average body weight. Groups 1-3 (n=10/group) served as treatment groups and were administered intermittent doses of PTH 1-34 at 1, 10, or 20 μg/kg of body weight. Group 4 (n=5) served as the untreated/vehicle control. All injections were administered subcutaneously, once per day for five consecutive days, followed by two days without injections. The treatment cycle was repeated three times, lasting 21 days. The control group (n=5) received injections of equivalent amounts of vehicle. On day 21, the animals were anesthetized with 5μl/g ketamine (20 mg/ml)/xylazine (2 mg/ml)/acepromazine (0.5 mg/ml) cocktail and blood was collected via cardiac puncture before euthanasia via cervical dislocation. The lumbar vertebrae, as well as the right and left hind limbs were harvested, cleaned of all soft tissue, and fixed for 24-48 hrs in 4% formaldehyde. This experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Pennsylvania State University College of Medicine.

A.3.4 Serum Analysis of bone turnover markers

Serum was separated from blood taken from fasted animals (~12 hrs) via centrifugation (30 min @ 1500 rpm) at 4°C. All serum was stored in single use aliquots at -80°C until analysis. Serum levels of mouse specific osteocalcin, TRACP 5b, type I collagen C-terminal telopeptides, and calcium were quantified by ELISA or colorimetric assay according to protocols provided by each manufacturer. For the quantification of osteocalcin, mouse sera were diluted 1/10 with sample buffer.
A.3.5 MicroCT Analysis

Specimens were harvested, fixed in 2% paraformaldehyde (48 hrs), stored in 70% ethanol, and then scanned using a vivaCT 40 Scanco Medical MicroCT (μCT) scanner (Brüttisellen, Switzerland). Slices were reconstructed with an isometric pixel size of 10.5 microns. Multiple regions within the trabecular bone of control specimens were evaluated to optimize guassian filter and threshold settings to 1 and 275 respectively, which were held constant throughout analysis. The distal femur was scanned and 75 slices proximal to the primary spongiosa of the growth plate were segmented by manually contouring the boundary between trabecular and cortical bone every 15 slices then morphing the intervening slices. The proximal tibiae were scanned and a region distal to the growth plate was segmented in a similar fashion. The fourth and fifth lumbar vertebrae were scanned and analyzed separately. The trabecular bone of each vertabra, excluding the cranial and caudal growth plates, were segmented as follows; two 50 slice stacks of each vertebra were manually contoured at the boundary between cortical and trabecular bone, every 10 slices, and morphed; the remaining slices between the two stacks were then morphed to produce a contiguous volume. The following indices were calculated using Scanco IPL software: bone mineral density (BMD, mg HA/ccm), bone volume (BV/TV), trabecular number (TbN, 1/mm), trabecular thickness (TbTh, mm), and trabecular separation (TbSp, mm).

A.3.6 Histochemical Demonstration of Alkaline Phosphatase Activity

After μCT analysis, all bones were decalcified in 10% EDTA, embedded in paraffin, and sectioned. Alkaline phosphatase (ALP) positive osteoblasts were identified in the distal femur employing a modified version of a method described by [215]. Sections were deparaffinized and rehydrated by passage through xylene and graded alcohol series to water,
then incubated at room temperature with 1% magnesium chloride in 0.01 M TRIS-maleate buffer (pH 9.2) for 2 hours. Sections were then incubated at 37°C for 50 min in freshly prepared ALP substrate (0.2 mg/ml napthol AS-MX phosphate and 0.4 mg/ml Fast Red TR in 0.01 M TRIS-maleate buffer, pH 9.2). After washing with distilled water, tissues were counterstained with aqueous hematoxylin (Vector laboratories, Burlingame, CA) and mounted with Vectamount.

A.3.7 Histochemical Demonstration of Tartrate-Resistant Acid Phosphatase Activity

Tartrate-resistant acid phosphatase (TRAP) activity was demonstrated in the distal femur as described by [216]. Briefly, deparaffinized and rehydrated tissue sections were incubated at room temperature in acetate-tartaric acid buffer (pH 5.0) for 20 min. Sections were then incubated at 37°C for 15-20 min in freshly prepared TRAP substrate (acetate-tartaric acid buffer, pH 5.0, containing 0.5 mg/ml Naphthol AS-MX phosphate and 1.1 mg/ml Fast Red TR). After washing with distilled water, tissues were counterstained with aqueous hematoxylin and mounted with Vectamount.

A.3.8 Quantification of Alkaline Phosphatase and Tartrate-Resistant Acid Phosphatase Positive Cells

The number of osteoblasts and osteoclasts appearing in digital images were captured with a SPOT RT cooled digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and counted within the primary spongiosa of the distal femur in a region of interest starting 1.0 mm proximal to growth plate and extended 1.0 mm in length and 3.0 mm medially from the periosteum. The number of cells was determined within four tissue sections spanning 120 microns of tissue. Data, quantified using Image J (NIH), are expressed as mean number of cells per mm of bone perimeter [217].
A.3.9 Statistical Analysis

One-way ANOVA with a Bonferroni post hoc test was performed using GraphPad PRISM version 5.0 (GraphPad Software, San Diego, CA) to compare all columns of data. All data are expressed as mean ± SD and were considered significant if p ≤ 0.05.

A.4 Results

A.4.1 Effects of Intermittent PTH 1-34 Dosing on SCID/Beige Body Weight

The thirty-five mice tolerated the study well. Throughout the experimental time course, the animals gained an average of 1.1 g (23.5 ± 1.32 g, mean initial and 24.6 ± 2.12 g, mean final of body weight). At no time during the study were differences in mean body weight observed between the different treatment groups.

A.4.2 Effects of Intermittent PTH 1-34 Dosing on Serum Bone Turnover Markers

To identify the dose effect of short term intermittent PTH 1-34 on bone turnover, four serum markers were quantified by ELISA or colorimetric assay. Osteocalcin, a marker of osteoblast activity, was enhanced by 30.3% (86.6 ± 29.41 ng/ml), and 14.9% (71.2 ± 10.5 ng/ml) in animals receiving 20 and 10 μg/kg of PTH 1-34 relative to control animals (56.3 ± 11.47 ng/ml, Fig 1A). Serum levels of osteocalcin in mice receiving 1 μg/kg of PTH 1-34 were not different relative to animals treated with vehicle. Serum mTRACP 5b levels, a marker of bone resorption, were similar for all treatment groups, and averaged 2.8 U/L (Fig 1B). Similarly, for all treatment groups the serum levels of two other bone resorption markers, calcium and C-terminal teleopeptides of type I collagen, were not different relative to animals treated with vehicle (Fig. 1C-D). The average total calcium level for all mice was approximately 3 mM (Fig 1D). These observations suggest that short term intermittent PTH
1-34 administration at doses between 10 and 20 µg/kg increase bone formation without enhancing bone resorption.

A.3 Effects of Intermittent PTH 1-34 Dosing on Trabecular Bone Architecture

To identify the dose effect of short term intermittent PTH 1-34 on cancellous bone architecture, the distal femur, proximal tibia, and fourth and fifth lumbar vertebrae were analyzed by μCT. The distal femur of mice administered 20 µg/kg of PTH 1-34 displayed increases in trabecular separation (24.6%), thickness (21.4%), and BMD (2.82%) with a concomitant decline in trabecular number (19.7%) relative to the distal femurs of mice treated with vehicle (Table 1A). Distal femurs of mice administered PTH 1-34 at concentrations of 10 µg/kg displayed similar directional changes; however, trabecular thickness was not different relative to controls (Table 1A). In the proximal tibias of mice administered 20 µg/kg PTH 1-34, a significant increase in trabecular thickness (14.7%) was observed (Table 1B). In mice administered PTH 1-34 at concentrations of 10 and 20 µg/kg, trabecular thickness in the fourth and fifth lumbar vertebrae was enhanced relative to controls, 8 and 13%, respectively (Table 1C-D). In the fourth and fifth lumbar vertebrae, trabecular number decreased (11% and 8%, respectively) in mice administered 20 µg/kg (Table 1C-D).

A.4.4 Effects of Intermittent PTH 1-34 on Osteoblast and Osteoclast Number

Consistent with serum levels of TRACP 5b, differences in the number of TRAP positive osteoclasts, across all treatment groups, compared to control animals were not observed (Figure 2). Conversely, the number of ALP positive osteoblasts demonstrated a dose dependent increase. Relative to vehicle treated animals, the number of ALP positive
osteoblasts increased by 28%, 46%, and 60% in animals receiving 1, 10 and 20 μg/kg of PTH 1-34, respectively (Figure 3E).
Figure A.1. Intermittent PTH 1-34 administration increases serum marker of bone formation. Three groups of young-adult male C.B-17 SCID/Beige mice (n=10/group) were administered PTH 1-34 intermittently at 1, 10, or 20 μg/kg/day. On day 21, serum levels of Osteocalcin (A); mTRACP 5b (B); Calcium (C); and Type I Collagen Telopeptides (D) were quantified by ELISA or colorimetric assay. Markers of bone loss were unchanged at all concentrations; however, serum osteocalcin, a marker of bone formation, was elevated in mice receiving 20 μg/kg/d relative to vehicle treated mice. *, Statistically significant compared to control, 0.
Figure A.2. Intermittent PTH 1-34 administration does not increase the number of tartrate-resistant acid phosphatase (TRAP) positive osteoclasts. Three groups of young-adult male C.B-17 SCID/Beige mice (n=10/group) were administered PTH 1-34 intermittently at 1, 10, or 20 μg/kg/day. On day 21, femurs were harvested, decalcified, embedded in paraffin, sectioned, and then stained for (TRAP). Columns represent the average number of TRAP+ cells (osteoclasts) per mm of bone perimeter in distal femur. 1, 10, and 20 correspond to the μg/kg of PTH 1-34 administered; 0, mice treated with vehicle alone.
Figure A.3. Intermittent PTH 1-34 administration increases the number of alkaline phosphatase (ALP) positive osteoblasts. Three groups of young-adult male C.B-17 SCID/Beige mice (n=10/group) were administered PTH 1-34 intermittently at 1, 10, or 20 μg/kg/day. On day 21, femurs were harvested, decalcified, embedded in paraffin, sectioned, and then stained for ALP (represented by yellow arrows). Panels A-D are representative images of ALP staining (Yellow arrows) in the distal femurs of mice treated with either vehicle (A); 1 μg/kg (B); 10 μg/kg (C); or 20 μg/kg/d (D) of PTH 1-34. Scale bar = 0.3 mm. Magnification = 20x. The columns in panel E represent the average number of ALP + cells (osteoblasts) per mm of bone perimeter in distal femur of mice administered increasing concentrations of PTH 1-34. *, Statistically significant compared to mice treated with vehicle only, 0.
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<th>BMD (mg HA/ccm)</th>
<th>Tb. N. (1/mm)</th>
<th>Tb.Sp. (mm)</th>
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**Table A.1 Effect of Intermittent PTH 1-34 on Cancellous Bone Remodeling.** MicroCT analysis of the distal femur, proximal tibia, and lumbar vertebrae of young-adult male C.B-17 SCID/Beige mice following three weeks of intermittent PTH 1-34 exposure. *n*, number of samples. Values are means ± SD; *p<0.05; *indicates significant differences from vehicle control.
A.5 Discussion

In this study three distinct measures of bone turnover were employed to identify the lowest dose of PTH 1-34 required to enhance bone formation without altering bone resorption in young-adult male SCID/Beige mice, following three weeks of intermittent administration. To our knowledge, we are the first to make these observations for the SCID/Beige murine model. Considered together, our endpoint observations suggest: 1) 20 µg/kg, compared to 1 and 10 µg/kg, is the dose of PTH 1-34 required to enhance bone formation in SCID/Beige mice over a three week time course; 2) PTH 1-34 enhanced bone formation did not require elevated catabolic/osteoclast activity; 3) PTH 1-34 administration did not enhance bone formation equally at all skeletal sites. Finally, since independent markers of bone formation were enhanced, but markers of bone resorption were unchanged, three weeks of intermittent PTH 1-34, administered at 20 µg/kg, enhances bone formation in SCID/Beige mice primarily by enhancing the number and activity of osteoblasts but not osteoclasts.

SCID/Beige mice were chosen for study because our laboratory currently employs this animal model in studies regarding the role of bone turnover in the colonization and establishment of human PCa in bone [74]. SCID/Beige mice demonstrate great efficiency for xenografting neoplastic human tissues, due primarily to their lack of natural killer cell activity, functional B and T lymphocytes, and macrophage defects that affect chemotaxis and motility (unpublished data, Jackson Laboratory). Since macrophage precursors arise from the same myeloid progenitor cells it is reasonable to speculate that SCID/Beige osteoclasts may also harbor defects that impede their functionality (i.e., ability to resorb bone). Although studies conducted to identify defects in osteoclasts have not appeared in the
literature, a report by Richard et al. [218] demonstrating significant bone loss in SCID/Beige mice as a consequence of hyperparathyroidism, i.e. a condition of chronically elevated PTH, indirectly suggests osteoclasts function normally in SCID/Beige mice. This conclusion is also supported by our unpublished observations demonstrating increased osteoclast number (TRAP staining) and cancellous bone loss (μCT) in hind limbs of SCID/Beige mice following three weeks of tail suspension. Thus, while SCID/Beige osteoclasts may still harbor defects, it is unlikely a functional impairment in their ability to resorb bone that contributes to our observations.

To our knowledge, the skeleton of SCID/Beige mice has not been previously characterized regarding its response to exogenous intermittent PTH 1-34. However, the SCID/Beige mouse was developed on a C57BL/6J background [219]; arguably the most widely employed mouse strain in studies of PTH action on bone [80, 205, 209, 210, 220, 221]. Our observations of increased serum osteocalcin, number of osteoblasts, and trabecular thickness are consistent with reports describing PTH 1-34 induced bone remodeling in C57BL/6J mice [80, 220]. However, our inability to detect any enhancement in osteoclast number and/or activity were surprising, especially when considering our μCT observations (increased trabecular separation and decreased trabecular number). This discrepancy may be attributed to the lower dose of PTH 1-34 and/or the timing of end stage analysis of bone turnover markers employed in this study. Elevations in bone resorptive parameters (osteoclast number and activity) have been reported when PTH 1-34 is administered intermittently, at doses of 40 µg/kg and higher, over a time course similar to the present investigation [74, 80, 210]. Herein the highest dose of PTH 1-34 administered was 2-4 times less than those typically administered; a dose that may be insufficient to increase osteoclast
number or activity. We are not aware of bone formation studies employing C57BL/6J mice that have employed low doses (< 20 μg/kg) of PTH 1-34 intermittently. However, bones of Swiss Webster and ICR mice have demonstrated anabolic responses to PTH 1-34, administered intermittently, at doses as low as 30 μg/kg without enhancing osteoclast number [84, 222]. The absence of detectable changes in osteoclast activity could also be due to the timing of serum bone turnover marker analysis. The analysis of sera two days following the last PTH 1-34 injection may have been too late to detect elevations in serum markers, a potential limitation of our approach. Indeed, morphologic changes in osteoclasts and elevations in serum calcium and collagen cross links have been reported to occur within min following a single PTH injection, and return to baseline following PTH 1-34 clearance [94, 223, 224]. While levels of calcium and collagen cross links in circulation may return to baseline following PTH clearance, our previous report [74] suggests elevations in serum TRACP 5b are detectable at least 2 days following the last PTH injection.

MicroCT analysis of cancellous bone architecture suggests the distal femur is more responsive to intermittent PTH 1-34 administration than either the proximal tibia or lumbar vertebrae, and that the anabolic action of intermittent PTH 1-34 is skeletal site specific. These observations are largely consistent with those of Iida-Klein et al. and Zhou et al. [80, 209]. Our observations also suggest lumbar vertebrae of SCID/Beige mice are responsive to intermittent PTH 1-34 administration but a robust response lags behind the distal femur. Differences in PTH 1-34 dose (40 μg/kg vs 20 μg/kg), study duration (7 vs 3 weeks), age (10 vs 8 wk old), strain (C57BL/6J vs SCID/Beige), and sex (female vs male) of mice employed may all contribute to the absence of robust cancellous bone changes in the proximal tibiae and lumbar vertebrae in this study. Low cancellous bone volume and mechanical loads in the
spine may also contribute [209, 225]. Indeed, humans and rodents differ in their responsiveness to PTH in the spine. Generally, the human spine is more responsive to intermittent PTH administration than that of the rodent [199, 226]. Moreover, an adequate number of trabeculae must also be present at the onset of PTH administration for maximal effect [209, 227]. PTH has been shown to augment cancellous bone mass when cancellous bone volumes (determined by histomorphometry) are greater than ~9% but not lower than 6% [82, 204, 227, 228]. Since cancellous bone volumes in rodents have historically been determined with histomorphometry, it’s difficult to compare our observations with those reported above, a limitation of our approach. Indeed, while treatment effects trend in the same direction, there is great disparity between the percent of cancellous bone volume identified by μCT compared to histomorphometry [228]. Unfortunately our laboratory is presently unable to perform bone histomorphometric analyses. Nevertheless, since the directional changes in cancellous bone architecture from the present investigation parallel those reported by Zhou and colleagues [209] it is tempting to speculate that the average cancellous bone volumes for the femur, tibia and lumbar vertebrae in SCID/Beige mice are similar to C57BL/6J mice, and thus correspondingly responsive to PTH; however, future investigations will have to conclusively determine this.

In the present study, histological analysis identified a 60% increase in the number of ALP positive osteoblasts in mice administered 20 μg/kg of PTH 1-34 compared to animals treated with vehicle alone. Serum osteocalcin, a marker of bone formation was similarly increased. Together, these data suggest intermittent PTH 1-34 administration increased osteoblast activity in SCID/Beige mice. A recent study by Bellido et al. [84] demonstrated the bone forming effects of intermittent PTH 1-34 administration involves the PKC signaling
pathway and ultimately leads to inhibition of osteoblast apoptosis in mice. Jilka and colleagues [83] have proposed that repeated bursts of survival signaling from intermittent PTH administration ultimately results in a reduction in apoptotic osteoblasts. While we did not measure osteoblast apoptosis, such a mechanism could explain the 60% increase in osteoblasts we observed, since just four injections of PTH 1-34 have been reported to reduce the number of apoptotic osteoblasts by 50% [84]. Other mechanisms that could contribute to the increased number of osteoblasts include: increased osteoblast progenitor differentiation and/or activation of bone lining cells. Dobnig and Turner [81] have reported that one week of daily PTH injections in rats enhances cancellous bone osteoblast number but not the percentage of osteoblasts that develop from progenitors. Recently similar conclusions were made by Jilka et al. [229] utilizing a murine model. Thus, these reports suggest that bone anabolism induced by short term intermittent PTH administration is not dependent upon increasing the number of osteoblast progenitors. While de-differentiation of bone lining cells back to osteoblasts may also contribute to increasing osteoblast number [230], they alone are unlikely to be responsible for the total increase in osteoblasts and osteocytes reported following intermittent PTH injections [82].

In conclusion, the current study is the first to identify the dose response of SCID/Beige mouse bone to intermittent PTH 1-34. Our observations suggest that 20 µg/kg of PTH 1-34, administered intermittently over a three week time course, is the dose required to enhance bone formation in young-adult male SCID/Beige mice. In contrast, bone resorption is unaltered by treatment. Although these observations, together, support our hypothesis, further investigation to identify the temporal, short term changes in serum calcium, serum phosphorous, endogenous PTH 1-84, static and dynamic bone
histomorphometry, as well as early and late stage markers of bone formation and resorption are warranted to address the limitations of this study, and to more thoroughly characterize the bone anabolic effects of low dose short-term intermittent PTH 1-34 in SCID/Beige mice.
Appendix B:

Additional Extracellular Calcium Experiments
Proliferation assays were also performed in calcium free DMEM to control absolute
calcium concentrations. Concentrations of 1.25 or 2.50 mM CaCl$_2$ were added to cells and
proliferation was measured every 24 hrs. Employing a modified Boyden chamber method,
cells were exposed to transient elevations in CaCl$_2$. This method employed transwell tissue
culture inserts and cells were seeded on the bottom of the tissue culture insert in order to
transfer cells to a higher concentration of calcium for 3 hrs/day. The results of these
experiments are below:

![Graph showing mean fluorescence over hours for 1.25 mM CaCl$_2$ and 2.50 mM CaCl$_2$.]

* indicates significantly different from control, p≤0.05

**Figure B.2 Transient exposure to elevated CaCl$_2$ enhanced proliferation of C4-2 cells.**

C4-2 cells were grown on the bottom of transwell tissue culture inserts in calcium free
DMEM and exposed to 1.25 or 2.50 mM CaCl$_2$ for 3 hrs/day. Every 48 hrs, cells were
removed from the bottom of the tissue culture insert and quantified with CyQuant dye. *
indicates significantly different from control, p≤0.05
Figure B.4 Transient exposure to elevated CaCl$_2$ enhanced proliferation of C4-2B4 cells. C4-2B4 cells were grown on the bottom of transwell tissue culture inserts in calcium free DMEM and exposed to 1.25 or 2.50 mM CaCl$_2$ for 3 hrs/day. Every 48 hrs cells were removed from the bottom of the tissue culture insert and quantified with CyQuant dye. * indicates significantly different from control, $p \leq 0.05$.
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Table B.1 Indicates changes in TRPV6, CaSR, and PI3K protein expression in response to continuous or transient increases in CaCl₂. Western blot analysis was performed with C4-2 and C4-2B4 cell lysates treated with 2.50 mM CaCl₂ for 3 hrs/day over a 24, 48, or 72 hr period.
References


74. Gomes, R.R., Jr., et al., Osteosclerotic prostate cancer metastasis to murine bone are enhanced with increased bone formation. Clin Exp Metastasis, 2009.


105. Xue, Y., et al., Exogenous 1,25-dihydroxyvitamin D3 exerts a skeletal anabolic effect and improves mineral ion homeostasis in mice that are homozygous for both the 1alpha-hydroxylase and parathyroid hormone null alleles. Endocrinology, 2006. 147(10): p. 4801-10.


Mosekilde, L., et al., The anabolic effects of human parathyroid hormone (hPTH) on rat vertebral body mass are also reflected in the quality of bone, assessed by biomechanical testing: a comparison study between hPTH-(1-34) and hPTH-(1-84). Endocrinology, 1991. 129(1): p. 421-8.


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