Purinergic Signaling in Bone As a Potential Mechanism in
Prostate Cancer Proliferation and Cancer-Induced Bone Pain

by

Michael Joseph Wilson

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Biological Sciences with Distinction

Spring 2017

© 2017 Michael Joseph Wilson
All Rights Reserved
Purinergic Signaling in Bone As a Potential Mechanism in
Prostate Cancer Proliferation and Cancer-Induced Bone Pain

by

Michael Joseph Wilson

Approved:

Mary E. Boggs, Ph.D.
Co-Advisor in charge of thesis on behalf of the Advisory Committee

Approved:

Randall L. Duncan, Ph.D.
Co-Advisor in charge of thesis on behalf of the Advisory Committee

Approved:

Kenneth L. van Golen, Ph.D.
Committee member from the Department of Biological Sciences

Approved:

Christopher J. Kloxin, Ph.D.
Committee member from the Board of Senior Thesis Readers

Approved:

Hemant Kher, Ph.D.
Chair of the University Committee on Student and Faculty Honors
ACKNOWLEDGMENTS

As part of this thesis, I would be mistaken to not acknowledge the people who have been instrumental during my journey to this point in time. First, I would like to thank my co-advisors Dr. Randall Duncan and Dr. Mary Boggs. Upon entering the Duncan lab in the 2015 spring semester, I was not entirely certain if research was something to become more involved with while attending the University of Delaware. Dr. Duncan and Dr. Boggs, you both nevertheless made me feel welcome and pushed me to learn and enjoy my time in the lab as a new experience. I am eternally grateful to both of you. Additionally, Dr. Boggs, you have proven instrumental in my journey towards becoming a better thinker and scientist overall. You have continually pushed me every day to think for better ideas in the areas of our research in order to become a well-rounded scientist. Dr. Boggs, you have greatly prepared me for what it will take to think like the future physician I wish to become and I will forever take pride in the lessons I have learned from you.

In addition to my co-advisors, I would like to thank all the friends I have met at the University of Delaware who have supported me through my journey in undergraduate research. Although there are too many to name, I want you all to know how grateful I am for the support. The University of Delaware has become a special place for me with many good memories and all of you will forever be a part of those.

Last, but not least, I would like to personally thank my parents, Mike and Linda Wilson, and my little sister Isabela. Dad and Mom, you both have been tremendous in your support of me and my goal to finish and present this thesis. You
both have taught me valuable lessons over my lifetime. You have taught and shown me how to become a person with character and have stressed the importance of integrity, honor, and work ethic. Isabela, you have been and remain the best little sister a big brother could ask for. As I have mentioned to you and others before, the best day of my life thus far was when you entered this world. Today, at just 13 years of age you are wise beyond your years and you have been very supportive of me through my journey at college and writing this thesis.

Dad, Mom, and Isabela, I love you all very much. Even in a thousand words, I could express the gratitude I have for your love and support. I will hold dearly onto each of your words of advice and support. All three of you have empowered me with the self-confidence and family support I need to achieve any goal. I look forward to drawing upon them again in my next journey at medical school.
TABLE OF CONTENTS

LIST OF FIGURES ............................................................................................................................. vi
ABSTRACT ......................................................................................................................................... vii

1 INTRODUCTION ................................................................................................................................. 1
  1.1 Rationale ....................................................................................................................................... 1
  1.2 Aim 1: Role of NGF, ATP, and Purinergic Signaling in Metastatic PCa Proliferation ......................... 2
  1.3 Aim 2: Role of ATP and Purinergic Signaling in Prostate Cancer-Induced Bone Pain (PCIBP) .......... 5
  1.4 A Hypothetical Model Displaying The Connection Between PCIBP and Proliferation of PCa in Bone ......................................................................................................................... 7

2 METHODS ....................................................................................................................................... 10
  2.1 Cell Culture of C4-2B4 Bone Derived Metastatic Prostate Cancer Cells ............................................. 10
  2.2 Hypotonic Swelling of C4-2B4s to Obtain Conditioned Media (CM) ................................................. 10
  2.3 Primary Cell Culture .................................................................................................................... 11
  2.4 Live Cell Imaging with DRGs and C4-2B4s ..................................................................................... 12
  2.5 Studies on ATP release from Hypotonically Swelled C4-2B4s ....................................................... 13
  2.6 Proliferation Studies of C4-2B4s ................................................................................................... 13
  2.7 Statistical Methods ....................................................................................................................... 14

3 RESULTS ....................................................................................................................................... 15
  3.1 Aim 1: Aim 1: Role of NGF, ATP, and Purinergic Signaling in Metastatic PCa Proliferation ............. 15
  3.2 Aim 2: ATP and Purinergic Signaling in Prostate Cancer-Induced Bone Pain (PCIBP) .................... 18

4 DISCUSSION .................................................................................................................................... 24

5 CONCLUSION ................................................................................................................................. 27

REFERENCES ...................................................................................................................................... 28
LIST OF FIGURES

Figure 1: Hypothetical model showing interactions between Osteogenic Cells, metastatic PCa, and nociceptors to produce PCIBP and promote PCa proliferation. ................................................................. 9

Figure 2: Effect of NGF on the Proliferation of C4-2B4s to determine the growth patterns of PCa in bone. Cell density was originally 100,000 cells/dish or 1818 cells/cm². All timepoints demonstrated an increase in proliferation from NGF treatment compared to control media. (n=1) ... 16

Figure 3: NGF supplemented media increased metabolism on average in C4-2B4s over 48 hours compared to control media. Apyrase reduced metabolism in C4-2B4s over 48 hours. (Number of trials is 3 for black and blue bars; Number of trials for green bar is 1; *p<.05) ............... 17

Figure 4: ATP released from hypotonically swelled C4-2B4s measured with a Luciferin-Luciferase Assay. C4-2B4s were hypotonically swelled for 0 minutes, 5 minutes, 10 minutes, and 15 minutes. 15 minutes of load released more ATP compared to other timed load amounts. ............... 20

Figure 5: [Ca^{2+}]_i Dose Response to CM in DRG from Hypotonically Swelled C4-2B4s to determine if CM from C4-2B4s contains the stimulus necessary to induce an intracellular calcium response in DRG. ............ 21

Figure 6: Application of CM from C4-2B4s on DRG with and without Apyrase to determine if the stimulus in Figure 5 that induced an [Ca^{2+}]_i response in DRG was ATP. (n=3; *p<.05) .................................................. 22

Figure 7: Maximum fluorescence intensity minus baseline in DRG with and without P2X3 inhibitor TNP-ATP when applied with CM from C4-2B4s. TNP-ATP determine if the P2X3 receptor is needed to induce an [Ca^{2+}]_i in DRG. (Error bars were not added to all data since n≠3 for each trial) ........................................................................... 23
ABSTRACT

Prostate cancer (PCa) is the third leading cause of cancer-related deaths in men in the United States. PCa preferentially metastasizes to bone and these metastases occur in approximately 2 out of 3 patients whose cancer has spread to other parts of the body. PCa that metastasizes to bone forms osteosclerotic tumors. Osteosclerotic tumors result from increased bone formation, but reduces bone strength resulting in increased mechanical strain in the bone microenvironment. The increased strain in bone resulting from tumor formation correlates with increased pain sensation. Prostate Cancer-Induced Bone Pain (PCIBP) is one of the most detrimental symptoms to patients with bone-localized cancer, greatly reducing patient’s quality of life. Current treatment for PCIBP is ineffective. In addition to PCIBP, PCa proliferates quickly once in the bone microenvironment, making it difficult to slow the disease progression and clinical outlook for the patient. Therefore, I seek to discover therapeutic targets that can elucidate why PCa proliferates quickly in bone and how this process and PCIBP could be linked. Discovering therapeutic targets could potentially increase the quality of life in patients with stage IV PCa.

In Aim 1, I propose that nerve-growth factor (NGF), a molecule implicated in PCa proliferation, enhances the potential for PCIBP by indirectly inducing PCa cell proliferation through upregulating purinergic receptors and sensitizing metastatic PCa cells to the mitogenic effects of ATP. To address the role of NGF in PCa proliferation and purinergic signaling, I applied NGF to C4-2B4 and performed cell counts as well as MTS cell proliferation assays. To determine the mitogenic properties of ATP on PCa cells, I added apyrase to the media to determine the effect of hydrolyzing ATP on C4-2B4 proliferation. NGF treatment demonstrated an increase in cell proliferation in
comparison to control media at all times tested. Addition of NGF with apyrase in an MTS assay showed a reduction in metabolism and thus proliferation over 48 hours. The proliferation studies indicate that NGF and ATP may play important roles together in the proliferation of metastatic PCa cells in bone.

In Aim 2, I proposed that mechanical load on PCa cells results in the release of ATP to activate local nociceptors through P2X3 receptors ultimately resulting in PCIBP. I mechanically loaded human bone-derived metastatic PCa cells, C4-2B4s, via hypotonic swelling. Resultant C4-2B4 load-response conditioned media (CM) +/- apyrase was applied to primary mouse dorsal root ganglia (DRG) and changes in intracellular calcium were measured. Apyrase is an enzyme that hydrolyzes ATP to AMP and should reduce the amount of ATP in the CM. To determine the role of P2X3 receptors in response to CM, TNP-ATP, a P2X3 specific antagonist was utilized. The data indicated that ATP in the CM of mechanically loaded C4-2B4 cells increased intracellular calcium in DRG which was reduced with apyrase. TNP-ATP did not significantly block response to CM suggesting another purinergic receptor may be responsible for this response.

These data overall indicate that proliferation of PCa and PCIBP could be linked through the mechanical release of molecules in bone from PCa and osteogenic cells. These data indicate purinergic receptors and NGF receptors as potential therapeutic targets for treating PCIBP.
Chapter 1
INTRODUCTION

1.1 Rationale

According to the American Cancer Society (ACS), prostate cancer (PCa) is estimated as the leading type of new cancer cases in US men in 2017. PCa is the third leading cause of cancer-related deaths in males. Furthermore, 2 out of 3 patients that are diagnosed with prostate cancer will have it metastasize, or spread, to other parts of the body (“Cancer Facts & Figures 2017” American Cancer Society). Bone is a common site of metastasis for prostate cancer due to its nutrient rich, low pH, high calcium, and especially highly vascularized environment. These traits of bone help promote the rapid growth of PCa (Muralidharan et al. 2013).

Metastatic PCa typically expresses an osteosclerotic phenotype resulting in abnormal bone formation. This abnormal bone formation results in the formation of weak woven bone which promotes an increase in mechanical load within the bone microenvironment (Muralidharan et al. 2013). As a result, stage IV PCa patients often experience severely debilitating bone pain resulting in mobility issues and decreased quality of life. Bone metastases like prostate cancer also results in pathologic fractures, hypercalcemia, and spinal cord compression due to the aggressive breakdown of the bone structure by the PCa lesions (“Signs and Symptoms of Bone Metastasis” American Cancer Society).

Presently there are no effective therapeutics for treating Prostate Cancer Induced Bone Pain (PCIBP). As noted by Chen et al. in their review, 75-90% of
patients with an advanced metastatic cancer will experience significant pain. Physicians, presently, often resort to using opioid based medications for the treatment of PCIBP. Opioids are regularly prescribed to patients with advanced stage cancers like stage IV PCa (Chen et al. 2013). Physicians have noticed that pain levels in cancer patients are not reduced even when levels of opioid use have approached the lethal dose limit. Additionally, hyperalgesia, a state in which nociceptors, the pain sensing neurons of the body, are sensitized from exposure to opioids has become increasingly common altogether proving the complexity of solving a clinically palliative problem like PCIBP (Lee et al. 2011).

As part of PCa disease progression there is the common use of molecules between cancer cell proliferation and PCIBP. Common molecules specifically ATP and NGF could be important to solving the clinical issue of both PCa cell proliferation and PCIBP. Ascertaining the role of these common molecules in cancer cell proliferation and PCIBP will be important in order to increase the quality of life in patients. My overall hypothesis for this project is: Molecules released by mechanically stimulated PCa cells in bone contribute to cancer cell proliferation and PCIBP.

I address this hypothesis in the following aims:

1.2 Aim 1: Role of NGF, ATP, and Purinergic Signaling in Metastatic PCa Proliferation

Previous evidence has suggested that growth factor pathways play a significant role in PCa metastasis to bone and that some of the factors and proteins involved in PCIBP play dual roles in the proliferation of PCa in bone. The change in growth factor
pathways through the PCa disease progression is regarded as the main contributing factor for PCa’s proliferation, adhesion, and motility (Muralidharan et al. 2013).

One growth factor, Nerve Growth Factor (NGF), is part of the neurotrophin family of peptides. This family includes other factors such as Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT-3), and Neurotrophin 4/5 (NT-4/5). Overall, neurotrophins play a broad role in survival, differentiation, neurite outgrowth, and maintenance of specific neuronal populations in the nervous system (Arrighi et al. 2010). NGF has been found to be expressed by osteoblasts. NGF expression was found to be the highest in osteoblasts that were mechanically loaded and exogenous NGF application increased bone formation (Tomlinson et al. 2017). These results indicate the importance of NGF in areas of remodeling in the bone microenvironment. Furthermore, NGF plays a significant role in the process of angiogenesis for PCa (Arrighi et al. 2010). From this evidence it is suggested that NGF mediated pathways could regulate metastatic PCa cancer growth once in the bone microenvironment.

The main receptor utilized by NGF to induce proliferation in PCa epithelia is the trkA or Tropomyosin-Related Kinase A receptor. The trkA receptor is a transmembrane glycoprotein belonging to the family of tyrosine kinase receptors. Binding of NGF to trkA causes dimerization of the receptor, activation of kinase activity, and then subsequent activation of various intracellular signaling pathways to induce proliferation (Arrighi et al. 2010). Normally, in the prostate epithelia, trkA operates in conjunction with p75 receptors to moderate cell proliferation and apoptosis. The p75 receptor is a member of the tumor necrosis factor death promoting receptor family and induces cell apoptosis when NGF is bound. As PCa progresses through its various grades and stages, p75 expression is lost, leaving only trkA
receptors (Arrighi et al. 2010). The loss of p75 eliminates any chance of programmed cell death in PCa and it is believed this downregulation of p75 expression is continued into the higher grades and stages of PCa metastasis.

In addition to NGF’s crucial role in binding with trkA. NGF also has been implicated in upregulating purinergic receptors. Experimental evidence has indicated that hindlimb muscles of mice, when treated with NGF, became sensitive to both α,β-meATP and ATP through upregulation of P2X3 receptors. Sensitivity to ATP in the hindlimb muscles was reduced when NGF was removed suggesting that NGF is crucial in dictating expression of P2X3 receptors (Liu et al. 2011). Furthermore, NGF treatment of PC12s, a cloned cell line from rat adrenal phaeochromocytoma, became sensitive to α,β-ATP and ATP by upregulation of seven P2X purinoceptors. Inward currents were measured in NGF treated PC12s indicating NGF-induced P2X receptors are functional channels (Sun et al. 2007). I believe that a similar action can be performed by metastatic PCa cells. Data has already eluded to ATP’s mitogenic effects in breast cancer particularly in causing proliferation of breast cancer cells through activation of purinergic receptors P2Y2 and P2Y4 (Jiang et al. 2015). With this data I believe that NGF potentially upregulates purinergic receptors and sensitizes PCa cells to the mitogenic effects of ATP and, thus, promotes proliferation.

In this first aim I hypothesize that **NGF indirectly induces proliferation in metastatic PCa cells through the upregulation of purinergic receptors. The upregulation of purinergic receptors sensitizes the metastatic PCa cells to the mitogenic effects of ATP.**
1.3 Aim 2: Role of ATP and Purinergic Signaling in Prostate Cancer-Induced Bone Pain (PCIBP)

ATP is the main energy source of cells, but has also been implicated in pain sensation (Jiang et al. 2015). I believe the increase in mechanical load and stress on bone from the continued proliferation of metastatic PCa is very important in the pathology of PCIBP. Increasing mechanical load in the bone microenvironment will potentially induce release of factors that increase pain levels further degrading the quality of life in patients. I seek to determine specifically in Aim 2 the role of ATP from PCa in neuronal excitability.

ATP is most often utilized to drive cellular processes and is used by proteins such as enzymes to carry out specific functions within cells. As of late, and of most importance to our project, ATP has been implicated in pain transmission as a potential neurotransmitter (Jiang et al. 2015).

In recent studies, ATP has been found to be a potent stimulator of pain sensation. Tumors have been found to release large amounts of ATP that accumulates more in the tumor interstitium than in healthy tissue (Pellegratti et al. 2008). Research to this point from multiple sources has shown that ATP can be released from mechanically loaded osteocytes. Dr. Mary Boggs displayed that the ATP from osteocytes can induce an intracellular calcium response in dorsal root ganglia (DRG) neurons (Boggs 2011). Since tumor cells release ATP as a mitogen, metastatic PCa cells could also perform a similar action in the bone microenvironment through mechanical load. My first sub-aim of this study was to determine if PCa cells release ATP through mechanical load and if that ATP can stimulate purinergic receptors on DRG.
In addition to determining whether ATP is released from metastatic PCa cells I wanted to ascertain how purinergic receptors play potentially into the pathology of PCIBP. Purinergic receptors are a large class of integral membrane proteins that play a large role in various cell types particularly for cell proliferation and apoptosis. There are two categories of purinergic or P2 receptors: P2X and P2Y. The P2Y receptors are G protein coupled receptors. The P2X channels are ionotropic cation selective ATP ligand gated channels. All P2X channels are equally permeable to sodium and potassium and significantly permeable to calcium (Falk et al. 2012). I believe the P2X3 receptor is important to my study because of previous research indicating that the P2X3 receptor has a large role in the presynaptic neuromodulation of afferent nerves and the release of excitatory neurotransmitter, glutamate (Falk et al. 2012). The P2X3 receptor is highly implicated in nociception, or pain signaling, and thus could be very important in the signal transduction involved in PCIBP. Furthermore, the P2X3 receptor is predominantly expressed in DRG making it feasible for us to study intracellular calcium responses in DRG to give a potential indication of neurotransmitter release (Falk et al. 2014).

Specifically, when studying the P2X3 channel, the ATP binding site requires three molecules of ATP for the pore to open. The EC50 for activating P2X3 receptors is 1 µM (Paukert et al 2001). This indicates that large amounts of ATP are needed in high quantities to activate the channel which tumor cells have been known to release. Additionally, my advisor, Dr. Mary Boggs, also showed in her Ph.D. dissertation that P2X3 receptors on dorsal root ganglia (DRGs) were readily activated by exogenous ATP at various concentrations: 0.1 µM, 1 µM, 10 µM, and 100 µM (Boggs 2011). Inhibition of P2X3 receptors in Dr. Boggs’ same study significantly reduced
intracellular calcium responses in DRGs when applied with exogenous ATP or ATP from osteocytes (Boggs 2011). These findings validated my reasoning behind studying the P2X3 receptor as a potential regulator of PCIBP.

From this evidence for Aim 2 I hypothesize that: **ATP from mechanically loaded metastatic PCa cells induces an intracellular calcium response in DRG through the P2X3 receptor.**

### 1.4 A Hypothetical Model Displaying The Connection Between PCIBP and Proliferation of PCa in Bone

Aims one and two display common factors that produce two pathologies linked to metastatic PCa in bone. There is the potential for the factors and proteins used in proliferation of PCa and PCIBP to be linked. Figure 1 is an adopted hypothetical model displaying what I believe is occurring in the bone microenvironment to produce PCIBP and proliferation of PCa.

As shown in the figure, I believe there is the presence of signaling that first promotes the proliferation of metastatic PCa cells and as a result promotes the occurrence of pain signaling to produce PCIBP. The figure shows through mechanical load the release of NGF from metastatic PCa cells and osteogenic cells. NGF from either cell type has the potential then to activate trkA receptors on metastatic PCa cells to increase proliferation through upregulation of purinergic receptors. This upregulation then gives more molecular targets for ATP to bind as a mitogen.

Additionally, NGF from either cell type has the potential to also induce axonal sprouting to increase pain sensation in nociceptors. Then, ATP through mechanical load is released from PCa cells and osteogenic cells to activate nociceptors in bone. These two converging signaling pathways have the potential to create a cycle that is
difficult to control because, as shown in the right side of the figure, it promotes: 1) an increase in PCa proliferation; 2) an increase in ATP release; and 3) an increase in neuron excitation. All three of these events further degrades the quality of life in the patient and is why I believe these molecular mechanisms serve a potential gateway in reversing or slowing the metastatic PCa disease progression.
Figure 1: Hypothetical model showing interactions between Osteogenic Cells, metastatic PCa, and nociceptors to produce PCIBP and promote PCa proliferation.
Chapter 2

METHODS

2.1 Cell Culture of C4-2B4 Bone Derived Metastatic Prostate Cancer Cells

C4-2B4s cells were a generous gift from Dr. Robert Sikes at the University of Delaware. C4-2B4s were cultured in T-75 flasks (Corning Cellgro; Manassas, VA) with Roswell Park Memorial Institution medium (RPMI) 1640-1X (Corning Cellgro; Manassas, VA) containing L-glutamine (300 mg/L) and HEPES (25 mM). Media was additionally supplemented with 1% penicillin-streptomycin (P/S), an antibiotic, (Hyclone; South Logan, UT) and 10% Fetal Bovine serum (Atlas Biologicals; Fort Collins, CO) were added to the RPMI media. Cells were grown to 60-80% confluency at 37 degree Celsius in 95% air, 5%CO₂ for 48 hours before use in experiments.

2.2 Hypotonic Swelling of C4-2B4s to Obtain Conditioned Media (CM)

C4-2B4s were grown to 60-80% confluency in 37 degree Celsius in 95% air, 5%-CO₂ for 48 hours or more, depending on cell density in the dish. C4-2B4s were then passaged using Cell Stripper, a non-enzymatic protein cell detacher to preserve cell surface proteins, (Corning, Manassas, VA) and placed into four T-25 flasks (Corning). Each flask corresponded to the amount of time the cells in each flask would be hypotonically swelled: 0 min, 5 min, 10 min, and 15 min. Once the C4-2B4s were at 60-80% confluent within each T-25, 2 milliliters of serum free RPMI media was added to the 5 min, 10 min, and 15 min flasks. 4 milliliters of serum free media was added to the 0 min flask since it was our control. After 1 hour of incubation in serum free media, 2 milliliters of sterile distilled water was added to the 5 min flask, 10 min flask, and 15 min flask. The 0 min flask did not receive any sterile distilled water.
Each flask was timed to ensure consistent amounts of hypotonic swelling. The 0 min T-25 received no hypotonic swelling since no amount of sterile distilled water was added. Hypotonic swelling was performed to mimic the increased mechanical load seen in bone from the bone structure’s breakdown due to metastatic PCa growth and proliferation. 3 milliliters of CM was removed from each flask and frozen down at -80 degrees Celsius for later use looking at intracellular calcium responses in dorsal root ganglia (DRG).

2.3 Primary Cell Culture

Dorsal Root Ganglia (DRGs) were isolated from adult male C57BJ/6J mice according to a University of Delaware IACUC-approved protocol and accepted practices following CO₂ asphyxiation and cervical dislocation. DRG were excised from L3-L5 vertebrae, rinsed in 1% P/S (Hyclone) in DMEM/F12 (Corning Cellgro) and then placed in culture media containing 10% Fetal Bovine Serum, P/S, and N1 (Sigma Aldrich; St. Louis, MO). N1 is a nutrient supplement which contains 0.5 mg/mL insulin from bovine pancreas, 0.5 mg/mL human transferrin, 0.5 ug/mL sodium selenite, 1.6 mg/mL putrescine, and 0.73 ug/mL progesterone. DRG were then cut into several large pieces and enzymatically broken down with collagenase (1000 U/mL; Sigma Aldrich) at 37 degrees Celsius for 25 minutes. DRGs are triturated carefully 15-20 times with a flame polished pipette and incubated to continue the tissue breakdown into single cells. Cells are then washed with DMEM/F12 and centrifuged for 5 minutes. Cells are triturated and washed an additional 4-5 times using the same steps as above to ensure single cells were extracted. Once the tissue was homogenously separated into single cells, DRGs were grown on Laminin coated
glass coverslips for eventual live cell calcium imaging. 10 uL of NGF were added to the DRG cell cultures to promote neurite and axonal growth.

2.4 Live Cell Imaging with DRGs and C4-2B4s

Live cell imaging was performed on primary DRG. Imaging was done with a calcium chelator, Fluo-4. Dissociated DRG neurons were first plated onto a laminin coated glass coverslip. After 48 hours of incubation, Fluo-4 (5 µM; Invitrogen; Carlsbad, CA) in .5% DMSO/Pluronic acid (Fisher Scientific; Waltham, MA) was loaded into the cells for a period of 10 minutes, depending on how well the cells absorbed the dye. The cells were then rinsed, covered, and rested in an incubator for an additional 10 minutes to ensure there was no excess Fluo-4 present. After the resting period, the loaded cells with the Fluo-4 imaged on a Zeiss 5 LIVE DUO under the Plan-Apochromat 10X water objective (filters 495-555) and images were taken every 50 ms for 4.6 minutes. Upon binding of calcium in the cytosol due to an intracellular calcium gradient change, Fluo-4 fluoresces by emitting a photon that is recognized by the specific wavelength. The amount of fluorescence is measured, and interpreted by a computer to show an individual response to an individual cell. Various stimuli including water, CM, antagonists, and agonists can be added to the cells to study their effects on baseline to peak response times. Maximum fluorescence over baseline is calculated to show rate of change in intracellular calcium from certain stimuli in DRG. Maximum fluorescence over baseline calculations can then be compared to other experiments to determine certain effects of agonists and antagonists.
2.5 Studies on ATP release from Hypotonically Swelled C4-2B4s

ATP released from hypotonically swelled C4-2B4s cells was measured using an ATP Bioluminescence Assay Kit (Roche Applied Sciences, Indianapolis, IN). As according to the protocol, ATP solutions were prepared by serial dilutions from $10^{-6}$ to $10^{-12}$ M in dilution buffer. ATP standards and experimental solutions were added into 96-well plate (Corning) at a volume of 50μL. Luciferase reagent was diluted to 100 μM in dilution buffer. 50 uL of Luciferase reagent was added to each standard and sample well in the 96-well plate using an automated injector. C4-2B4s were hypotonically swelled with sterile water for 0, 5, 10, and 15 minutes. Bioluminescence was read after each period of hypotonic swelling. Bioluminescence in the C4-2B4s was measured using a FLUOstar Optima plate reader (BMG Labtechnologies, Ortenberg, Germany) after a 1s delay from addition of luciferase reagent. Quantification was performed by subtracting the blank wells from the raw data; ATP concentrations were determined by preparing standard curve (Boggs 2011).

2.6 Proliferation Studies of C4-2B4s

In order to study the effects of NGF on C4-2B4 proliferation assays were performed. Both MTS cell proliferation and cell counting assays were utilized. For our cell count experiments, C4-2B4s were plated onto 100 mm tissue-culture treated petri dishes (Corning; Corning, NY) at 1818 cells/cm². NGF (Gibco) was applied to specified dishes at 50 ng/uL. Every 24 hours of incubation the number of cells were counted on each dish. Cell numbers were averaged and graphed to compare NGF treatment versus serum reduced control media. Apyrase (10 U/mL; Sigma Aldrich; St. Louis MO) was added ± NGF test the effect of hydrolyzing ATP to AMP.
In addition to the cell counting assays, an MTS assay was used. An MTS assay is a colorimetric sensitive quantification of cells for proliferation or cytotoxicity assays. In our case, for proliferation, we wanted to determine the effects of NGF on the proliferation of C4-2B4s. As a result of the cancer cell’s metabolism, MTS tetrazolium compound is reduced by the cells to formazan which has a purple color. This is thought to be done by NAD(P)H-dependent dehydrogenase enzymes in cells that are metabolically active. The amount of formazan produced can be quantified by a plate reader and read at an absorbance of 450 nm. The absorbance gives an indication of metabolic rate and thus proliferation. C4-2B4s were plated at a concentration of 3,106 cells/cm$^2$ on a 96 well plate. 200 uL of media is added to each well and the cells are incubated for 12 hours in media containing FBS (10%). After incubation for 12 hours in serum-containing media, experimental media, which contained RPMI 1640 1X plus 50 ng/mL NGF, 1% FBS, and 1% P/S, was added for 24, 48, 72, or 96 hours. After running incubating in experimental media, 40 uL of MTS Reagent was added to each well and incubated for 1 hour at 37 degrees Celsius. After incubating with the MTS reagent for 1 hour, the 96 well plate was placed onto a plate reader and the absorbance at 450 nm was taken. Each absorbance was adjusted from to blank wells containing serum free RPMI to take account for the presence of phenol red in the media. Average absorbance for each experiment was plotted versus the control.

## 2.7 Statistical Methods

One way analysis of variance with Tukey-Kramer posthoc tests were used to determine the statistical significance of differences between our experimental groups. Significance was defined by a $p$ value <0.05.
Chapter 3

RESULTS

3.1 Aim 1: Role of NGF, ATP, and Purinergic Signaling in Metastatic PCa Proliferation

In Aim 1 I sought to elucidate the mechanisms in the proliferation of PCa in bone. I wanted to ascertain whether NGF, a protein commonly found in bone to support neuronal maintenance, axonal growth and nociceptor sensitization, could indirectly induce C4-2B4 metastatic PCa cell proliferation. I hypothesized that NGF indirectly induces proliferation in C4-2B4s through upregulation of purinergic receptors. The upregulation of purinergic receptors sensitizes the C4-2B4s to ATP and its mitogenic effects. To determine the role of NGF in C4-2B4 cell proliferation, NGF was applied to C4-2B4s. Cell counts were completed every 24 hours to determine NGF’s proliferative effects. As shown in Figure 2, application of NGF onto C4-2B4s over 96 hours increased proliferation. The starting cell density was 100,000 cells/dish or 1818 cells/cm² at 0 hours. At 24 hours of treatment NGF increased proliferation by 19% compared to control media. At 48 hours of treatment NGF increased proliferation by 12% compared to control media. At 72 hours of treatment NGF increased proliferation by 9% compared to control media and by 96 hours NGF increased proliferation by 25% again compared to control media.

In continuing in my goal of determining the proliferative effects of NGF, I utilized an MTS assay. The MTS assay was used instead of cell counting to provide more accurate data regarding the effects of NGF on proliferation. My goal with the MTS assay was to determine the change in metabolic rate of C4-2B4s as a result of NGF treatment over 48 hours. The change in metabolic rate indirectly indicates proliferation through the production of formazan. I applied NGF at 50 ng/µL over 48
hours for three separate trials. As seen in Figure 3, application of NGF over 48 hours, showed an increase in metabolism. Statistical calculations demonstrated significant difference between the 48 hour control and the 24 hour control and NGF. Significance was also obtained between the 48 hour NGF and the 48 hour control, 24 hour NGF, and 24 hour control. Next, I applied Apyrase at 10 U/mL and saw a reduction in proliferation over 48 hours as shown in Figure 3.

Figure 2: Effect of NGF on the Proliferation of C4-2B4s to determine the growth patterns of PCa in bone. Cell density was originally 100,000 cells/dish or 1818 cells/cm². All timepoints demonstrated an increase in proliferation from NGF treatment compared to control media. (n=1)
Figure 3: NGF supplemented media increased metabolism on average in C4-2B4s over 48 hours compared to control media. Apyrase reduced metabolism in C4-2B4s over 48 hours. (Number of trials is 3 for black and blue bars; Number of trials for green bar is 1; *p<.05)
3.2 Aim 2: ATP and Purinergic Signaling in Prostate Cancer-Induced Bone Pain (PCIBP)

For Aim 2, I determined the role of ATP and purinergic signaling in neuron excitability with the potential for regulating PCIBP. The purpose of the study was to determine if ATP and purinergic signaling, a mechanism used in the proliferation of metastatic PCa from Aim 1, is utilized by C4-2B4s to excite DRG in order to promote PCIBP signaling. C4-2B4 cells, a highly metastatic prostate cancer cell line, were hypotonically swelled for 0 min, 5 min, 10 min, and 15 min. As seen in Figure 4, C4-2B4s release varying concentrations of ATP after certain periods of hypotonic swelling inducing mechanical strain. I utilized Luciferin-Luciferase Bioluminescence to quantify ATP release from C4-2B4s. The data from this study showed an increase in ATP release across 5, 10, and 15 minutes of hypotonic swelling. From these data, I again utilized hypotonic swelling to induce ATP release from C4-2B4s for future application to DRG. The 0 min of hypotonic swelling served as my control. The hypotonic swelling simulated the mechanical strain seen in bone from the growth of metastatic PCa cells and the breakdown of the tissue. After 60 seconds of baseline readings in fluo-4 loaded DRG, a 10 min CM set from C4-2B4s was applied to study DRG intracellular calcium responses. DRG larger than 25 µm in diameter were excluded from the study as they are not associated with nociception. I noted upon application of the CM a large fluorescence peak as shown in Figure 5. Following the peak fluorescence in the DRG, I saw the response return to baseline readings.

Due to the response seen within DRG from the application of C4-2B4 CM, I sought to determine if the stimulus shown to be active in Figure 5 was ATP. Thus, I applied CM, again extracted from hypotonically swelled C4-2B4s, and applied it to DRG. To determine if ATP was the stimulus, I applied Apyrase to our CM. Apyrase
hydrolyzes ATP down to AMP. Upon application of CM after 60 seconds of baseline reading, an intracellular response was noted and, this time, was recorded as maximum fluorescence intensity over baseline. This measurement is used to show the change in the fluorescence intensity upon application of the stimulus. After initial control readings with control media, Apyrase was mixed in with the CM sets specifically the 0 min, 5 min and 10 min sets and applied to Fluo-4 loaded DRG neurons. Fluorescence was measured and maximum fluorescence intensity was found to be reduced over all time point sets (0 min, 5 min, and 10 min) of CM. Significance was seen in each CM set without Apyrase compared to control. Statistical significance was also seen between the 10 min B4 CM and 10 min B4 CM+Apyrase as shown in Figure 6.

After determining that ATP from CM of C4-2B4s was a valid stimulus in inducing intracellular calcium responses in DRG my next step in my study was to ascertain if purinergic receptors, specifically the P2X3 receptor, was the receptor used in DRG to induce an intracellular calcium response to ATP in C4-2B4 CM. I again applied 0 min, 5 min, or 10 min CM sets from hypotonically swelled C4-2B4s to DRG. On different trials I pre-loaded DRG with P2X3 specific inhibitor: TNP-ATP. As seen in Figure 7, a reduction in intracellular calcium responses was seen between the 10 min B4 CM vs 10 min+TNP set. All other CM sets (0 min and 5 min) did not show a reduction in the intracellular calcium response in DRG.
Figure 4: ATP released from hypotonically swelled C4-2B4s measured with a Luciferin-Luciferase Assay. C4-2B4s were hypotonically swelled for 0 minutes, 5 minutes, 10 minutes, and 15 minutes. 15 minutes of load released more ATP compared to other timed load amounts.
Figure 5: $[\text{Ca}^{2+}]_i$ Dose Response to CM in DRG from Hypotonically Swelled C4-2B4s to determine if CM from C4-2B4s contains the stimulus necessary to induce an intracellular calcium response in DRG.
Figure 6: Application of CM from C4-2B4s on DRG with and without Apyrase to determine if the stimulus in Figure 5 that induced an [Ca^{2+}]_i response in DRG was ATP. (n=3; *p<.05)
Figure 7: Maximum fluorescence intensity minus baseline in DRG with and without P2X3 inhibitor TNP-ATP when applied with CM from C4-2B4s. TNP-ATP determine if the P2X3 receptor is needed to induce an \([Ca^{2+}]_i\) in DRG. (Error bars were not added to all data since n≠3 for each trial)
Chapter 4

DISCUSSION

Overall, these data implicate many points in my project. First, I have demonstrated in Aim 1 the ability of NGF and ATP through purinergic signaling to act as co-factors in the proliferation of metastatic PCa cells. In Aim 2, I demonstrated that ATP and purinergic signaling are able to also promote neuronal excitability in DRG through the P2X3 receptor. The data in Aim 2, has the implication \textit{in vivo} that ATP and the P2X3 receptor could contribute in producing action potentials in nociceptors necessary for inducing pain signaling for PCIBP. Overall these data particularly indicate the probability of NGF, ATP, and purinergic signaling playing dual pathological roles. NGF and ATP through purinergic signaling can simultaneously promote neuronal outgrowth and excitability as well as continued proliferation of metastatic PCa (Arrighi \textit{et al.} 2010). My data does not indicate if proliferation from NGF and ATP is from binding of trkA or purinergic receptors. As part of my future studies, I will further investigate those receptors.

Additionally, my data has broad implications surrounding the function of osteogenic cells. Osteogenic cells have been known to release factors that could contribute to PCa cell proliferation and PCIBP. In referencing back to Figure 1, the factors and proteins within the bone microenvironment, namely ATP, NGF, and purinergic receptors, are all commonly used by native bone cells particularly osteocytes and osteoblasts to maintain bone structure and function (Boggs 2011; Genetos \textit{et al.} 2005; Mogi \textit{et al} 2000). Data to this point has proven that osteocytes release ATP and osteoblasts release both ATP and NGF (Boggs 2011; Genetos \textit{et al.} 2005; Nakanishi \textit{et al.} 1994). Tying these already known facts regarding the use of
those factors in osteogenic cells could prove useful in understanding the complexity behind metastatic PCa’s interactions with the bone microenvironment.

Furthermore, the common utilization of these ATP, NGF, and purinergic signaling by all cell types, as shown in Figure 1, creates a vicious cycle within the bone microenvironment. This vicious cycle serves solely to promote the survival and health of metastatic PCa cells through a molecular crosstalk within bone. I believe the activation of multiple processes at once by NGF, ATP, and purinergic signaling quickly degrades the quality of life in patients. This vicious cycle quickly becomes uncontrollable which makes it difficult to treat therapeutically.

I seek in future studies to understand how the proliferation of PCa cells in bone could be directly linked to PCIBP. To truly understand how these two processes, connect into a vicious cycle more studies will need to be completed to ascertain whether our general hypothesis holds true. Studies on the effect of NGF binding to trkA receptors in metastatic PCa cells to promote proliferation in vitro will prove useful in truly understanding if NGF induces proliferation over a total of 96 hours. I will also need to complete studies looking at the upregulation of purinergic receptors by NGF to determine if NGF increases the sensitivity of metastatic PCa cells to ATP. By showing how sensitive C4-2B4s are to ATP after NGF treatment I can determine how readily ATP is used as a mitogen by metastatic PCa cells in bone. Additionally, the effect of ATP from mechanically loaded osteocytes on metastatic PCa cells will prove useful in revealing whether ATP from osteocytes could inadvertently contribute to the aggressive proliferation of PCa in bone. Of equal importance will be studying the effect of NGF from mechanically loaded osteoblasts on metastatic PCa proliferation. There is a large body of evidence stating the importance of NGF in bone
formation in osteoblasts. Elucidating the relationship between osteoblast NGF and metastatic PCa cell proliferation will be useful in connecting the different cell types within bone. On another note, determining the role of the P2X3 receptor in neuronal excitability will be necessary for truly understanding how purinergic receptors and ATP could promote PCIBP signaling.

As part of a broader future study goal, completion of animal studies on the functions of metastatic PCa in vivo will be largely important to this project. Specifically, my goal with this future work is to study pain related behaviors in mice with metastatic PCa in the presence of certain inhibitors. In these studies, I can potentially utilize purinergic receptor inhibitors, for example, directly into areas of bone to study their effectiveness at attenuating pain related behaviors. I can also potentially utilize apyrase to study the effect of hydrolyzing ATP in the bone of mice with metastatic PCa. The use of apyrase may reveal the importance of ATP in PCIBP and ultimately reduce pain related behaviors. Lastly, performing these future animal studies on mice with metastatic PCa will be useful in studying cell to cell interactions between native cells in bone with metastatic PCa. I can utilize certain antibodies to stain whole tissue mounts of mouse tibias to determine certain cell interactions that indicate active processes in proliferation and/or PCIBP.
Chapter 5

CONCLUSION

My overall hypothesis was that molecules released by mechanically stimulated PCa cells in bone contribute to cancer cell proliferation and PCIBP. In Aim 1 I hypothesized that NGF indirectly induces proliferation in metastatic PCa cells through upregulation of purinergic receptors. This upregulation sensitizes the metastatic PCa cells to the mitogenic effects of ATP. My data in Aim 1 indicated that NGF could induce proliferation over 96 hours. Repeat experiments looking at NGF did show this same effect over 48 hours and application of Apyrase to hydrolyze ATP reduced proliferation indicating ATP’s importance in this process. In Aim 2, I hypothesized that ATP released from mechanically loaded metastatic PCa cells induces an intracellular calcium response in dorsal root ganglia (DRGs) through the P2X3 receptor. My data indicated that CM from mechanically loaded C4-2B4s contained the stimulus necessary to induce an intracellular calcium response in DRG. Also, my data indicated that ATP can induce neuronal excitability through an intracellular calcium response in DRG. Furthermore, my data potentially implicates the P2X3 receptor as the receptor used by ATP to induce an intracellular calcium response in DRG.

These data indicate that NGF, ATP, and purinergic signaling play dual roles in the pathology of stage IV PCa. The use of these factors in proliferation and promoting PCIBP indicates the likelihood of a linkage between metastatic PCa cell proliferation and PCIBP. I believe NGF and purinergic receptors can serve as potential therapeutic targets that can effectively treat both metastatic PCa proliferation and PCIBP.
REFERENCES


Liu, Jiahao, Jialiu David Li, Jian Lu, Jihong Xing, and Jianhua Li. "Contribution of Nerve Growth Factor to Upregulation of P2X3 Expression in DRG Neurons of Rats


