

Electronic Thesis and Dissertation Repository

---

11-22-2016 12:00 AM

## Role of Lipid Mediators in P2X7 Signaling in Osteoblasts

Erin Eyer, *The University of Western Ontario*

Supervisor: Dr. Jeffrey Dixon, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Clinical Science degree in Orthodontics

© Erin Eyer 2016

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Orthodontics and Orthodontology Commons](#)

---

### Recommended Citation

Eyer, Erin, "Role of Lipid Mediators in P2X7 Signaling in Osteoblasts" (2016). *Electronic Thesis and Dissertation Repository*. 4361.

<https://ir.lib.uwo.ca/etd/4361>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact [wlsadmin@uwo.ca](mailto:wlsadmin@uwo.ca).

## ABSTRACT

P2X7 is a cell-surface nucleotide receptor that plays a critical role in skeletal mechanotransduction; however, the signaling pathways mediating these effects are poorly understood. Previous studies showed that the nucleotide analog benzoylbenzoyl-ATP induces anabolic gene expression in osteoblastic cells. Our first objective was to determine whether this effect was mediated by P2X7. Inhibition of anabolic gene expression by a P2X7-specific antagonist established involvement of this receptor. Our second objective was to investigate the role of lipid mediators, lysophosphatidic acid (LPA) and prostaglandin (PG), both of which are produced in response to P2X7 activation. The effect of P2X7 on expression of the anabolic gene *Ptgs2* was abolished by an LPA receptor antagonist or inhibition of PG synthesis. Furthermore, in the absence of nucleotide, LPA and PGE<sub>2</sub> synergistically stimulated *Ptgs2* expression. Thus, LPA and PG signaling pathways appear to be necessary and sufficient to mediate the effect of P2X7 on *Ptgs2* expression.

## KEYWORDS

Bone remodeling, orthodontic tooth movement, mechanotransduction, adenosine 5'-triphosphate (ATP), cyclooxygenase-2 (COX-2), osteoblasts, purinergic P2 receptors, purinergic signaling, purinoceptor, P2X, P2X7, *P2rx7*, real-time reverse transcription-polymerase chain reaction (RT-PCR), gene expression, A438079, *Ptgs2*, *Dmpl*, *c-Fos*, MC3T3-E1 cells

## ACKNOWLEDGMENTS

Completing this thesis would not have been possible without the assistance of many individuals. First, I would like to extend gratitude to Dr. S. Jeffrey Dixon, my supervisor. Thank you for your patience, guidance, encouragement and for humbly offering your scientific expertise. All of your time and effort is greatly appreciated.

I would like to thank the members of my committee. Dr. Antonios Mamandras and Dr. Ali Tassi. I am grateful to you both for believing in me and giving me the opportunity to pursue a career in orthodontics. I feel extremely privileged to have been part of this program. I would also like to thank Dr. Bruce Hill and Dr. Frank Beier for volunteering their valuable time and constructive feedback.

Thank you to the many members of the Dixon and Sims lab, who had an instrumental role in helping me to complete this thesis. Special thanks go to Ryan Beach for assisting me with numerous lab techniques, contributing to my thesis with your expertise in Corel Draw and ensuring that supplies were always on hand. I am eternally grateful to Dr. Meena Na, who introduced me to all necessary lab techniques, dedicated many hours to train me and gave me guidance every step of the way. To Dr. Yara Hosein, Dr. Noelle Ochotny, Dr. Kim Beaucage, Brandon Kim, and Dr. Alex Pereverzev thank you for your enthusiasm and constructive comments towards the project and for your camaraderie. Last but not least, I'd like to thank Dr. Stephen Sims for all of your guidance and constructive feedback when I needed it. Your dedication to students and the scientific profession is truly inspiring.

Thank you to all my fellow residents, past and present, for all of your support and for making these past three years unforgettable.

I would also like to thank my future father-in-law, Dr. Dan Sullivan, for your much needed guidance and encouragement in entering the amazing field of orthodontics.

Your experience, wisdom and levelheaded advice humbles me daily and I feel extremely fortunate to have your continued support.

To my parents: I cannot thank you enough. Your endless love, reassurance and countless sacrifices have allowed me to conquer my dreams. Thank you for instilling in me the need to strive to accomplish goals through hard work and to never settle for average.

Lastly, my sincerest gratitude goes to my partner in life, Konnor. Thank you for your continuous support and for all of your sacrifices in allowing me to pursue my dreams. I could not have endured this journey without your love, logic and humor in times where I needed it most. I am so excited to share many more of life's adventures with you in our future together.

## TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGMENTS .....	iv
TABLE OF CONTENTS.....	vi
LIST OF ABBREVIATIONS.....	ix
1 Orthodontic Tooth Movement.....	1
2 Bone cells .....	2
2.1 Osteoblasts .....	2
2.2 Osteoclasts .....	4
2.3 Osteocytes .....	5
3 Regulation of Bone Remodeling and Mechanotransduction .....	6
4 Mechanotransduction and Orthodontic Tooth Movement .....	7
5 P2 Receptors .....	7
5.1 P2X7 Receptor .....	9
5.1.1 P2X7 Receptor Signaling in Osteoblasts .....	11
5.1.2 P2X7 signaling in osteoclasts .....	14
6 Lysophosphatidic Acid.....	15
7 Prostaglandins .....	16
8 Rationale, Hypothesis and Objectives of the Research.....	18
MATERIALS AND METHODS.....	24
1 Materials and Solutions.....	24
2 Cell Culture .....	24
3 RNA Isolation .....	24
4 Real-Time RT-PCR Analyses .....	25

5 Statistical Analysis .....	26
RESULTS .....	30
1 BzATP-induced stimulation of <i>Ptgs2</i> (COX-2) expression was inhibited by the P2X7 antagonist, A438079 .....	30
2 BzATP-induced stimulation of <i>Dmp1</i> expression was inhibited by A438079 .....	30
3 BzATP-induced stimulation of <i>c-Fos</i> expression was inhibited by A438079.....	31
4 BzATP-induced stimulation of <i>Ptgs2</i> expression was inhibited by treatment with VPC-32183 and ibuprofen .....	31
5 PGE <sub>2</sub> and LPA had a synergistic stimulatory effect on <i>Ptgs2</i> gene expression in MC3T3-E1 cells.....	32
DISCUSSION.....	38
1 Summary and Conclusions.....	38
2 P2X7 regulates expression of anabolic genes .....	39
3 Role of lipid signaling pathways in mediating effects of P2X7.....	40
4 Limitations of the study and suggestions for future studies.....	42
5 Relevance of the study in orthodontic tooth movement.....	43
REFERENCES .....	46
APPENDICES .....	57
CIRRICULUM VITAE .....	60

## LIST OF FIGURES

Figure 1. Proposed P2X7-LPA/PG signaling axis promoting osteogenesis. ....	13
Figure 2. Effects of ATP stimulation of P2X7 receptors in osteoblasts and osteoclasts. ....	14
Figure 3: Conversion of phospholipids to prostaglandin (PGE <sub>2</sub> ). ....	17
Figure 4: Schematic showing the potential effects of a P2X7 antagonist, A438079, on BzATP-induced gene expression at the P2X7 receptor. ....	20
Figure 5: Investigating the roles that PG and LPA may play downstream of P2X7 receptor activation. ....	21
Figure 6: Effect of PG (PGE <sub>2</sub> ) and LPA on gene expression in osteoblastic cells. ....	22
Figure 7: The proposed P2X7-PG-LPA axis and its effects on gene expression in osteoblast precursor cells. ....	23
Figure 8. Timeline of the first series of experiments. ....	27
Figure 9. Timeline of the second series of experiments. ....	28
Figure 10. Timeline of the third series of experiments. ....	29
Figure 11: BzATP-induced stimulation of <i>Ptgs2</i> expression was completely inhibited by the P2X7 antagonist A438079. ....	33
Figure 12: BzATP-induced stimulation of <i>Dmp1</i> expression was completely inhibited by A438079. ....	34
Figure 13: BzATP-induced stimulation of <i>c-Fos</i> expression was completely inhibited by A438079. ....	35
Figure 14: BzATP-induced stimulation of <i>Ptgs2</i> expression was inhibited by the COX inhibitor ibuprofen and the LPA receptor antagonist VPC-32183. ....	36
Figure 15: PGE <sub>2</sub> and LPA combined induced <i>Ptgs2</i> gene expression in MC3T3-E1 cells. ....	37
Figure 16: Summary of the findings. ....	45



## LIST OF ABBREVIATIONS

$\alpha$ MEM	$\alpha$ -minimum essential medium
ADP	adenosine 5'-diphosphate
ALP	alkaline phosphatase
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
ATF-4	activating transcription factor 4
ATP	adenosine 5'-triphosphate
BMP	bone morphogenetic protein
BSP	bone sialoprotein
BzATP	2',3'- <i>O</i> -(4-benzoylbenzoyl)ATP
cAMP	cyclic adenosine monophosphate
COL1	collagen type 1
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
FBS	heat-inactivated fetal bovine serum
FGF	fibroblast growth factor
IGF	insulin-like growth factor
IL	interleukin

KO	knockout
LPA	lysophosphatidic acid
LPAR	lysophosphatidic acid receptor
M-CSF	macrophage colony stimulating factor
MAPK	mitogen-activated protein kinase
NF- $\kappa$ B	nuclear factor-kappa B
NFATc1-4	nuclear factor of activated T cells, cytoplasmic 1-4
NSAIDs	non-steroidal anti-inflammatory drugs
OCN	osteocalcin
OPG	osteoprotegerin
OPN	osteopontin
OSX	osterix
OTM	orthodontic tooth movement
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PGE <sub>2</sub>	prostaglandin E <sub>2</sub>
PKA	protein kinase A
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PLC	phospholipase C
PTH	parathyroid hormone

RANK	receptor activator of nuclear factor kappa B
RANKL	RANK ligand
RT-PCR	reverse transcription polymerase chain reaction
RUNX2	runt-related transcription factor 2
SEM	standard error of the mean
SOST	sclerostin
TGF	transforming growth factor
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate

## LIST OF APPENDICES

Appendix A: Results presented without the normalization as percentage of maximum gene expression. ....	57
Appendix B: Permission to use Figure 2. ....	59

## INTRODUCTION

### 1 Orthodontic Tooth Movement

Orthodontic tooth movement is the result of a complex interaction between an applied force and a physiologic response within the periodontal supporting tissues. Prolonged forces placed on teeth are responsible for remodeling the adjacent bone. The cellular processes by which orthodontic induced bone remodeling occurs have been well described; however, the initial events of mechanotransduction, the process by which mechanical stimuli are transformed into biological responses, are unclear. Ultimately, understanding mechanisms by which mechanotransduction arises may permit more efficient orthodontic tooth movement.

A theoretical model of tooth movement is comprised of three stages: (1) initial compression of tissues, resulting in an alteration in blood flow associated with pressure and tension within the periodontal ligament; (2) the initiation and release of chemical messengers, such as cytokines and prostaglandins (PGs), and (3) further activation of cells mediating osteogenesis and bone resorption (Proffit, 2012). Within four hours of sustained force application, levels of cyclic adenosine monophosphate (cAMP) increase significantly (Proffit, 2012). cAMP is a second messenger that regulates the differentiation of osteoclasts (bone removing cells) and osteoblasts (bone forming cells) (Roberts, 1989). In addition, levels of prostaglandins (PGs), interleukin-1 beta (IL-1 $\beta$ ) and nitric oxide (NO) rise in the periodontal ligament (PDL) (Krishnan and Davidovitch, 2006).

The “Pressure-Tension Theory” of orthodontic tooth movement was developed following classic histologic research in orthodontics (Krishnan and Davidovitch, 2006; Schwarz, 1932). The application of a force to a tooth creates both an area of compression and tension within the PDL. This leads to an alteration in blood flow and development of chemical signals triggering downstream cellular responses. On the compression side, osteoclastic activity prevails and bone resorption ensues. Immediate precursors of

osteoclasts are present in the PDL and are activated to mature once appropriate signals have directed them. Additionally, new osteoclasts can be recruited to the PDL from hematopoietic organs via the blood stream and alveolar bone marrow cavities (Proffit, 2012). On the tension side, osteoblasts are activated directly and bone deposition results. The coordinated coupling of osteoblasts and osteoclasts is essential to orthodontic tooth movement (Krishnan and Davidovitch, 2009). Light orthodontic forces are preferable, since they promote frontal resorption and efficient tooth movement through bone, whereas heavy forces lead to necrosis of the PDL and undermining resorption (Proffit, 2012). Undermining resorption results in delayed tooth movement, since the necrotic area must be completely removed by osteoclasts before tooth movement can occur.

## 2 Bone cells

Three main types of bone cells regulate bone metabolism: osteoblasts (bone forming cells), osteoclasts (bone resorbing cells) and osteocytes (terminally differentiated osteoblasts). These cells respond to various environmental signals, including biological and mechanical stimuli, to elicit specific responses based on the physiologic needs of bone. Receptors on the surface of bone cells bind exogenous signals and transmit this information to the nucleus where gene expression is initiated (Feldman, 2013).

### 2.1 Osteoblasts

Osteoblasts are derived from mesenchymal stem cells that can also differentiate into other cell types such as chondrocytes and fibroblasts (Minguell et al., 2001). Osteoblasts carry receptors that respond to chemical mediators, which play a central role in bone metabolism (Feldman, 2013). In order to commit to the osteoblast lineage, a complex series of transcriptional events must be initiated and maintained by a number of factors including: bone morphogenetic proteins (BMPs), transforming growth factor-beta (TGF- $\beta$ ), Wnt signaling pathways, insulin-like growth factors (IGFs) and parathyroid hormone (PTH) (Huang et al., 2007). Runt-related transcription factor-2 (RUNX2) is a critical transcription factor responsible for osteoblast differentiation (Huang et al., 2007). Early and late stages of osteoblast differentiation are controlled by Osterix (OSX), which acts downstream of RUNX2. The combined effects of RUNX2 and OSX lead to the

development of a preosteoblast, which expresses type I collagen and bone sialoprotein (BSP). Development of the mature osteoblast is dependent upon stimulation of activating transcription factor 4 (ATF-4) with Wnt/ $\beta$ -catenin signaling components. The mature osteoblast expresses type I collagen, alkaline phosphatase and osteocalcin (OCN), which are necessary for the formation of osteoid and its subsequent mineralization (Harada and Rodan, 2003; Robling et al., 2006).

Differential gene expression is important in each stage of development for the osteoblast. Genes required for the initial activation and proliferation (*c-Fos*, *c-Jun*, and *c-Myc*) and cell cycle progression (histones and cyclins) are expressed in pre-osteoblasts (Feldman, 2013). Other factors critical to this stage of osteoblast development are: fibroblast growth factor (FGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), insulin-like growth factor-1 (IGF-1), BMPs, and collagen type I (COL1) (Feldman, 2013).

Proliferation of osteoblasts is followed by production and organization of the extracellular matrix (ECM). In this stage, collagen secretion and cross-linking predominates. Genes involved with skeletal ECM mineralization (e.g. ALP) are expressed.

The third stage is characterized by a reduction in genes produced in the proliferation stage and an increase in hydroxyapatite (HA) production in the ECM. Non-collagenous proteins with HA-regulating abilities (osteopontin (OPN), osteocalcin (OCN) and bone sialoprotein (BSP)) are expressed during this stage of osteoblast development (Feldman, 2013).

An organic matrix (osteoid), composed of type I collagen, non-collagenous proteins and proteoglycans, is secreted by mature osteoblasts. Deposition of calcium phosphate mineral leads to mineralization of the osteoid. As bone formation continues, osteoblasts become incorporated into the mineralized osteoid and terminally differentiate into osteocytes. Osteocytes communicate with one another and osteoblasts via gap junctions. These intercellular connections are thought to be critical for transmitting signals resulting from the mechanical stimulation of bone (Bonewald, 1999). Osteocytes express a distinct

group of genes, including sclerostin (*Sost*), matrix extracellular phosphoglycoprotein (*Mepe*), and dentin matrix protein-1 (*Dmp1*) (Feng et al., 2003).

The final stage in osteoblast development serves as an editing function for modifications needed in the ECM (Feldman, 2013). Once bone formation is complete, the majority of osteoblasts undergo apoptosis. The remaining cells join the osteocyte population or become bone-lining cells (Clarke, 2008).

Jones et al. described the first responses in osteoblasts after application of a mechanical stress. The first events are an increase in intracellular free calcium concentration and hyperpolarization of the membrane potential through the activation of potassium channels. Phospholipase C (PLC) is activated to release inositol triphosphate. PLC is thought to allow a positive feedback mechanism in order to keep the mechanosensitive channels open by further activation of protein kinase C (PKC). Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is then activated, acting on the phospholipid membrane to release arachidonic acid, leading to the production of prostaglandins (PGs) within about 10 minutes. This is followed by the release of products from the lipoxygenase pathway that ultimately lead to an increase in cAMP. cAMP mediates phosphorylation reactions in the nucleus and cytoplasm of the osteoblast, regulating downstream gene expression and bone remodeling (Jones et al., 1991).

More recently, osteoblasts have been shown to release ATP in response to mechanical stimulation (Romanello et al., 2001; Romanello et al., 2005; Buckley et al., 2003; Genetos et al., 2005).

## 2.2 Osteoclasts

Mononucleated precursors of the monocyte/macrophage lineage fuse to form multinucleated osteoclasts (Novack and Teitelbaum, 2008). Two key signaling molecules are essential to the formation, resorptive activity and survival of osteoclasts: macrophage-colony-stimulating factor (M-CSF) and receptor activator of NF- $\kappa$ B ligand (RANKL) (Nobuaki et al., 1998). Both M-CSF and RANKL are produced in membrane-bound and soluble forms by marrow stromal cells and osteoblasts (Boyce and Xing, 2008; Boyle et al., 2003). Osteoprotegerin (OPG), is a decoy protein that binds to



RANKL, and thus inhibits the binding of RANKL to its receptor RANK (Boyce and Xing, 2008; Boyle et al., 2003). In binding to RANKL, this decoy protein leads to inhibition of osteoclast function and induces osteoclast apoptosis. The function of osteoclasts is further mediated by complex interactions among several signaling pathways such as those activated by PTH, calcitonin, vitamin D, tumor necrosis factor (TNF), estrogen and several interleukins (Ericksen et al, 1986; Feldman, 2013). PTH, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and vitamin D upregulate RANKL on the surface of osteoblasts, and thereby stimulate osteoclast activation (Kale et al., 2004; Feldman, 2013).

In order for the osteoclast to begin resorption, it must attach to the bone surface and activate proton pumps along its ruffled border. In the acidic environment of the resorption lacunae, dissolution of bone mineral is possible. Osteoclasts secrete proteases such as cathepsin K to degrade the organic matrix comprised mainly of type I collagen (Novack and Teitelbaum, 2008). Interleukins (IL-1 $\beta$ , IL-6) and PGE<sub>2</sub> encourage osteoclast resorption (Brough et al., 2003). Once resorption is complete, the osteoclast may migrate to a new site on the bone surface to continue resorbing bone or it may undergo apoptosis (Novack and Teitelbaum, 2008).

### 2.3 Osteocytes

Osteocytes are the most abundant bone cells; however, their function is poorly understood. Some osteoblasts become incorporated in the osteoid as it is produced, becoming osteocytes. Since osteocytes are connected to one another by long cell processes, they are thought to be involved in receiving mechanical signals and transmitting these stimuli to other cells in bone (Feldman, 2013).

Osteocytes synthesize sclerostin, a protein responsible for acting in a paracrine fashion to suppress bone formation. Sclerostin, a product of the *SOST* gene, binds to LRP5/LRP6 receptors to inhibit the Wnt signaling pathway and thus reduces bone formation (Robling and Turner, 2009). Both PTH and mechanical loading inhibit the effects of sclerostin (Turner and Pavalko, 1998; Harada and Rodan, 2003; Robling and Turner, 2009).

### 3 Regulation of Bone Remodeling and Mechanotransduction

Osteoblasts and osteoclasts are responsible for maintaining a fine balance between bone formation and resorption in bone remodeling. Several critical local and systemic factors, including PTH, insulin-like growth factors (IGFs), estrogen and bone morphogenetic proteins (BMPs), along with mechanical stimuli, coordinate activity of bone cells to control skeletal homeostasis (Harada and Rodan, 2003). Resorption and formation are strictly coupled under physiological conditions; an imbalance may contribute to bone loss in a variety of skeletal disorders such as osteoporosis and inflammatory diseases including periodontitis and rheumatoid arthritis (Novack and Teitelbaum, 2008). Bone remodeling is a necessary element in orthodontic tooth movement.

Each remodeling cycle is initiated by osteoclast precursors, which form osteoclasts that remove old bone over a period of 2-4 weeks. Preosteoblasts are recruited to the site after a reversal phase and bone formation occurs with osteoid secretion and subsequent mineralization (Grol et al., 2009). Bone formation occurs over a period of about 4-6 months (Baron, 2003).

Bone homeostasis is regulated by differential gene expression in bone cells. Local and systemic factors regulate bone cell activity by activating specific intracellular signaling pathways. Once exogenous signals have been identified by receptors bound to the cell membrane, activation of the receptor and subsequent transduction of the signal to the cell's nucleus occurs. In the cell nucleus, transcription factor complexes bind to specific DNA promoter sequences, resulting in the expression of specific genes.

Several signal transduction pathways have been implicated in bone formation. For instance, the  $\text{Ca}^{2+}$ -NFAT signaling pathway has been shown to regulate osteoclast formation (Boyle et al., 2003, Novack and Teitelbaum, 2008; Teitelbaum and Ross, 2003). In addition, this pathway has a significant role in the regulation of osteoblast formation and function (Koga et al., 2005; Sun et al., 2005). The Wnt/ $\beta$ -catenin signaling pathway is important in maintaining a balance between osteoblast and osteoclast activity. This pathway induces gene expression to upregulate differentiation of osteoblast

precursor cells and it increases the OPG to RANKL ratio, thus inhibiting osteoclast formation (Issack et al., 2008; Kubota et al., 2009).

#### 4 Mechanotransduction and Orthodontic Tooth Movement

Mechanotransduction is the process by which mechanical stimuli are transformed into cellular responses. For over one hundred years, it has been known that bone remodels in response to physical forces (Wolff, 1892). Mechanical loading increases bone formation, resulting in improved skeletal strength. Disuse, conversely, suppresses bone formation and accelerates bone resorption (Duncan and Turner, 1995; Robling et al., 2006). More recently, research has shown that mechanotransduction involves nucleotide release and downstream P2 receptor activation (Robling et al., 2006; Dixon and Sims, 2000).

Osteoblasts and osteocytes, communicating via gap junctions, may act as sensors of these mechanical signals to initiate bone remodeling. While the exact mechanism of mechanotransduction is poorly understood, it is known that mechanical strain contributes to alterations in gene expression (Robling et al., 2006). It is thought that mechanical loading causes bending in bone and stimulates ATP release from osteoblasts, osteoclasts and osteocytes. ATP binds to P2 purinergic receptors on the cell surface of bone cells to initiate signaling that results in osteoclastogenesis and osteoblast differentiation to stimulate bone remodeling (Robling et al., 2006).

Orthodontic tooth movement is made possible by skeletal mechanotransduction. Orthodontic forces induce cells in the periodontal ligament and alveolar bone to release signals that stimulate bone remodeling via alterations in gene expression (Krishnan and Davidovitch, 2006). Since mechanotransduction involves nucleotide release and activation of purinergic receptors, these may have important effects on orthodontic tooth movement.

#### 5 P2 Receptors

P2 receptors are a group of cellular receptors that bind extracellular nucleotide messengers, such as ATP and ADP. The P2 receptors are subdivided into P2X and P2Y receptors based on their mechanism of activation and structural differences (Burnstock,

1976; Burnstock and Kennedy, 1985; Burnstock, 2004; Burnstock, 2007). P2X receptors are ATP-gated ion channels (non-selective for Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup>). Adenine nucleotide binding activates these receptors and leads to membrane depolarization and influx of intracellular Ca<sup>2+</sup>. There are seven types of P2X receptors (P2X1-P2X7) that are differentially expressed in multiple cell types, including both peripheral and central neurons, smooth muscle cells and neuroendocrine cells (Dixon and Sims, 2000). The eight receptors in the P2Y family of receptors (P2Y1, 2, 4, 6, 11-14) are G protein-coupled receptors that are activated by adenine and/or uridine nucleotides (Dixon and Sims, 2000).

ATP is released from numerous cell types, including osteoblasts, in response to mechanical disturbance and hypoxia (Bodin and Burnstock, 2001; Lazarowski et al., 2011; Lazarowski, 2012). Released nucleotides may act in an autocrine or paracrine manner to influence local P2 receptor signaling (Orriss et al., 2012). Furthermore, nucleotide signaling through P2 receptors may be responsible for mechanotransduction in bone remodeling (Burnstock, 2004; Orriss et al., 2010).

The function of extracellular nucleotides in regulating osteoblastic activity is unclear (Dixon and Sims, 2000). ATP has been found to stimulate proliferation of murine calvarial cells, MC3T3-E1 (Suzuki et al., 1993). Additionally, in MC3T3-E1 cells, ATP has been shown to activate phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (Suzuki et al., 1995; Panupinthu et al., 2007; Panupinthu et al., 2008), contributing to metabolism of arachidonic acid and the subsequent production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (Watanabe-Tomita et al., 1997).

The elevation of cytosolic free Ca<sup>2+</sup> concentration in osteoblasts has been implicated in the initial signaling events leading to stimulation of osteoclastic activity (Guggino et al., 1989). In addition, our lab has shown that nucleotides may act on cells of the osteoblast lineage to enhance the resorptive actions of PTH (Dixon and Sims, 2000).

Both P2X and P2Y receptors have also been identified on osteoclasts. Activation of these receptors through nucleotide binding has shown to increase osteoclast activity (Morrison et al., 1998). In addition, nucleotides regulate osteoclast numbers through the

induction of apoptosis as a result of the activation of the P2X7 receptor (Dixon and Sims, 2000).

### 5.1 P2X7 Receptor

The P2X7 receptor is unique among P2X receptors in both its structure and the fact that it requires a 10-fold higher concentration of ATP (>100  $\mu$ M) to be activated. Of the P2X family, the P2X7 receptor is the largest, with a length of 595 amino acids. It contains two hydrophobic membrane-spanning regions separated by a long glycosylated extracellular ATP-binding domain (Suprenant et al., 1996; Chessell et al., 2005). The P2X7R also has a long cytoplasmic C-terminal tail, responsible for mediating its interaction with other proteins (North, 2002). Whereas ATP is the most potent agonist of other P2XR subtypes, 2',3'-*O*-(4-benzoylbenzoyl) ATP (BzATP) is a more potent agonist of the P2X7 receptor than ATP (Jacobson et al., 2002).

The P2X7 receptor is an ATP-gated ionotropic channel that plays an important role in inflammation, pain and bone adaptation responses (Volonte et al., 2012; Chessell et al., 2005; Lister et al., 2007; North, 2002; Labasi et al., 2002; Grol et al., 2009). P2X7 become active (opens) after binding extracellular ATP, which can be released from adjacent cells under mechanical strain (Bodin and Burnstock, 1998). Initial activation of P2X7 receptors by ATP leads to an opening of non-selective cation channels, resulting in an influx of  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  and an efflux of  $\text{K}^{+}$ . Prolonged activation of P2X7 with ATP leads to large pore formation that allows for passage of large hydrophilic molecules within seconds (North, 2002). ATP activation also induces downstream signaling events, which are dependent upon cell type, extracellular conditions and the concentration of extracellular ATP (Burnstock, 2007).

In humans, several genetic differences in the P2X7 receptor exist and these can affect channel function. Single nucleotide polymorphisms (SNPs) of the P2X7 receptor have been identified to be associated with various musculoskeletal, inflammatory and cardiovascular diseases (Jiang et al., 2013). Among the effects of loss of function in P2X7 is enhanced loss of bone mineral density in post-menopausal women (Gartland et al., 2012). Furthermore, variability in function of the P2X7 receptor may explain

individual differences in response to orthodontic forces and may explain why some individuals are susceptible to root resorption (Viecilli et al., 2009; Hartsfield, 2009).

Key mediators of inflammation, such as PGE<sub>2</sub>, IL-1 $\alpha$ , and IL-1 $\beta$  are released in response to P2X7 activation. All of these factors are critical in the maintenance of bone physiology (Ferrari et al., 2006; Li et al., 2005). The release of cytokines, in turn, can induce accumulation of neutrophils and lymphocytes (Chen and Brosnan, 2006). Neutrophils further assist in mediating the inflammatory response by acting to eliminate apoptotic cells from the site and prevent further necrosis. Labasi et al. showed that murine macrophages deficient in *P2rx7* (the gene encoding P2X7) do not release IL-1 in response to stimulation with ATP, resulting in an attenuated acute inflammatory response (Labasi et al., 2002).

Due to the role of P2X7 in mediating inflammatory pain, several pharmaceutical companies have initiated a search for selective P2X7 receptor antagonists. Systematic administration of the selective P2X7 antagonist A438079 reduces thermal hyperalgesia (Nelsen et al., 2006; Donnelly-Roberts and Jarvis, 2007). Progress made recent years strongly suggests that receptor-specific P2X antagonists may be useful analgesics in humans. Additionally, such drugs may modulate bone remodeling, such as in orthodontic tooth movement.

Investigating *P2rx7* knockout (KO) mice has provided significant insight into this receptor's role in mechanotransduction. P2X7-deficient mice have reduced sensitivity to mechanical loading due to decreased secretion of PGE<sub>2</sub> (Li et al., 2005). Viecilli et al. found that lack of the P2X7 contributed to slower removal of hyalinized tissue in *P2rx7* KO mice. In addition, these KO mice exhibited increased rates of external root resorption in orthodontically moved teeth (Viecilli et al., 2009). Furthermore, P2X7 KO mice also show decreased periosteal bone formation and increased trabecular bone resorption (Ke et al., 2003). Collectively, these findings suggest that P2X7 may be necessary for skeletal growth and may contribute to physiologic balance between bone formation and resorption in response to mechanical force. Clearly the P2X7 receptor is an ideal candidate for mediating orthodontic responses, which involve both the induction

of an acute inflammatory response and bone remodeling (Roberts, 1989; Reitan, 1994; Roberts et al., 2004).

A number of cell-type specific signaling pathways are associated with P2X7 receptor activation. Because these pathways are cell-specific, it appears that in each cell type P2X7 has unique downstream signaling effects.

#### 5.1.1 P2X7 Receptor Signaling in Osteoblasts

ATP and BzATP induce opening of the P2X7 non-selective cation channel, leading to an increase in intracellular  $\text{Ca}^{2+}$ . This then couples to multiple signaling pathways in cells of the osteoblast lineage (Grol et al., 2009).

First, P2X7 receptors have been shown to mediate extracellular signal-regulated kinase (ERK) 1/2 activation by fluid shear stress in an osteoblast-like cell line (Liu et al., 2008). ERK activation by fluid flow was found to stimulate expression of various osteogenic genes including: osteopontin (OPN), *c-Fos*, cyclooxygenase-2 (COX-2), collagen type I, and *cbfa1/RUNX2* (You et al., 2001; Okumura et al., 2008; Wadhwa et al., 2002; Mehrotra et al., 2006). Furthermore, Rubin et al. (2003) found that physiologic levels of mechanical strain utilized ERK 1/2 kinase to regulate production of NO and RANKL in a manner that promotes net bone formation (Rubin et al., 2003).

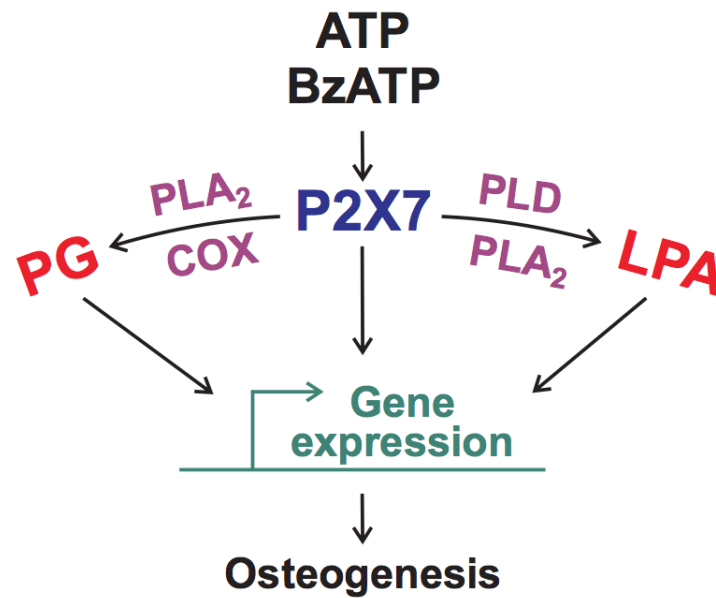
Second, the P2X7 receptor is responsible for fluid shear-stress-induced activation of the transcription factor nuclear factor kappa-B (NF- $\kappa$ B) (Genetos et al., 2011). MC3T3-E1 osteoblasts express the NF- $\kappa$ B inhibitory protein I $\kappa$ B $\alpha$  under static conditions. Under fluid shear stress, I $\kappa$ B $\alpha$  levels decreased and nuclear localization and expression of NF- $\kappa$ B occurs. Genetos et al. showed that P2X7R-mediated activation of NF- $\kappa$ B occurs independently of lysophosphatidic acid (LPA) signaling. Chen and Brosnan demonstrated that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) release requires purinergic signaling and that the NF- $\kappa$ B is required for maximal cyclooxygenase-2 (COX-2) induction in response to fluid shear stress (Chen and Brosnan, 2006).

Third, Panupinthu et al. reported that P2X7 receptors signal through phospholipase D (PLD) and phospholipase A<sub>2</sub> (PLA<sub>2</sub>). Two products are the result of PLD and PLA<sub>2</sub>

activity: lysophosphatidic acid (LPA) and arachidonic acid. Arachidonic acid is metabolized to PGs and other eicosanoids. LPA acts on its receptors on osteoblasts to cause membrane blebbing via a pathway dependent on Rho-associated kinase. The P2X7-LPA-PG axis is thought to be of significance to P2X7-mediated osteogenesis during mechanotransduction (Figure 1). Panupinthu et al. proposed that mechanical stimuli induce release of ATP, which then acts through P2X7 receptors on osteoblasts, leading to the production of prostaglandins and LPA. Prostaglandins and LPA then act in an autocrine or paracrine manner to enhance bone formation, explaining the role of the P2X7 receptors in skeletal development and mechanotransduction (Panupinthu et al., 2007; Panupinthu et al., 2008).

Dr. Na (Na, 2016) investigated the effects of dexamethasone, a corticosteroid with anti-inflammatory effects, on gene expression in MC3T3-E1 cells following P2X7 receptor activation with BzATP. She showed that BzATP stimulation of genes *Ptgs2* and *Dmp1* was inhibited by dexamethasone. This finding further exemplifies the role that P2X7 may play in regulating osteoblast development and eventual bone formation during orthodontic tooth movement and shows that dexamethasone may inhibit these processes (Na, 2016).



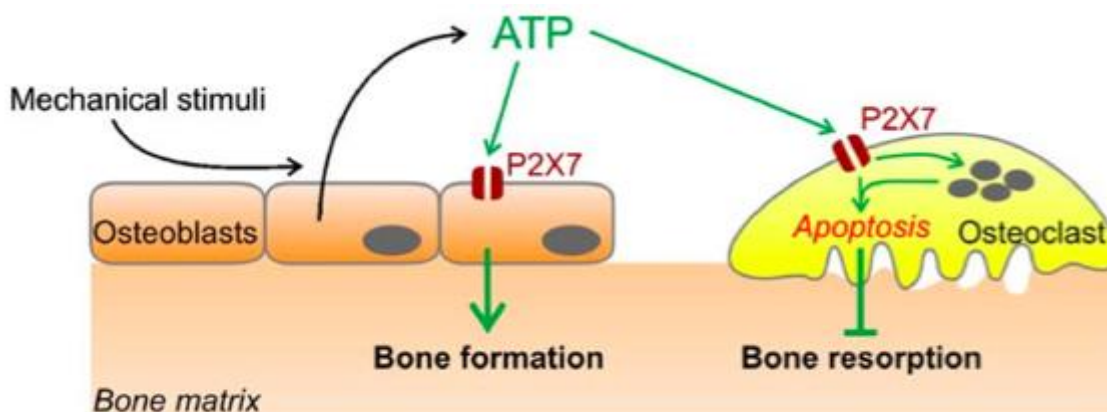


**Figure 1. Proposed P2X7-LPA/PG signaling axis promoting osteogenesis.**

Activation of P2X7 by endogenous ATP or exogenous BzATP leads to production of lysophosphatidic acid (LPA) and prostaglandin (PG). LPA is synthesized from membrane lipids following the activation of phospholipase D (PLD) and phospholipase A<sub>2</sub> (PLA<sub>2</sub>). Activation of PLA<sub>2</sub> also leads to the production of arachidonic acid that is converted to PGs by the action of cyclooxygenase (COX). LPA and PG may act in an autocrine or paracrine manner to induce expression of anabolic genes and subsequently promote osteoblast differentiation and mineralization (osteogenesis). Image modified with permission from Panupinthu et al., 2008.

### 5.1.2 P2X7 signaling in osteoclasts

Initial studies revealed that P2X7 may be involved in osteoclast fusion and up-regulating osteoclast differentiation (Gartland et al., 2003). However, further investigation into P2X7R KO mice revealed that P2X7 in osteoclasts has a role in initiating apoptosis and thus inhibits osteoclast-induced bone resorption (Ke et al., 2003; Grol et al., 2009). These conflicting results have led to the explanation that the timing and degree of P2X7 receptor activation may govern its effects on osteoclasts.



**Figure 2. Effects of ATP stimulation of P2X7 receptors in osteoblasts and osteoclasts.**

This figure illustrates potential roles for P2X7 signaling in osteoblasts and osteoclasts. Nucleotides, including ATP, released from osteoblasts in response to mechanical stimulation signal through P2 receptors to mediate bone remodeling. P2X7 activation in osteoblasts results in bone formation, whereas its activation in osteoclasts promotes apoptosis. Image reprinted with permission from Purinergic Signaling, Grol et al., 2009; Appendix B.

## 6 Lysophosphatidic Acid

Lysophosphatidic acid (LPA) is a potent bioactive phospholipid produced by several cells such as activated platelets. LPA is present in low levels in the plasma under resting conditions and becomes elevated in inflammation and tissue injury. LPA acts on six G protein-coupled receptors (LPA<sub>1-6</sub>) (Yung et al., 2014; Sheng et al., 2015). Activation of LPA receptors can lead to elevation of intracellular Ca<sup>2+</sup> concentration, activation of ERK and stimulation of phosphatidylinositol 3-kinase signaling (PI3K) (Sims et al., 2013). LPA is also involved in regulating cell migration and chemotaxis (Sugimoto et al., 2006).

LPA induces the production of cytokines in osteoblasts, which are known to regulate osteoclast function. Our lab has shown that osteoblasts produce LPA, which may be involved in the pathogenesis of bone diseases, such as in rheumatoid arthritis (Sims et al., 2013). Activation of P2X7 receptors on osteoblasts leads to the production of LPA, through the sequential activation of PLD and PLA<sub>2</sub>. LPA may act on osteoblasts in an autocrine manner to regulate osteoblastic activity. Osteoblast-derived LPA may also act in a paracrine manner to regulate osteoclasts (Sims et al., 2013).

LPA has a stimulatory effect on osteoclastogenesis. However, it is not known if this effect is direct or if LPA affects other cell types that may in turn alter osteoclast behavior. Lapierre et al. showed that LPA enhances the survival of osteoclasts in vitro, promotes their fusion and suppresses their apoptosis (Lapierre et al., 2010). LPA acts through several receptor types on osteoclasts to induce an increase in intracellular Ca<sup>2+</sup> and activate the critical transcription factor NFATc1, which is associated with RANKL-stimulated osteoclast differentiation (Pereverzev et al., 2008). LPA also has a direct effect on osteoclast motility, specifically by retraction of osteoclast pseudopodia (Lapierre et al., 2010). Effects of LPA on osteoclast resorption has produced conflicting results, as some studies have shown a slight decrease in resorption by osteoclasts (Lapierre et al., 2010) and others have shown an increase (McMichael et al., 2010).

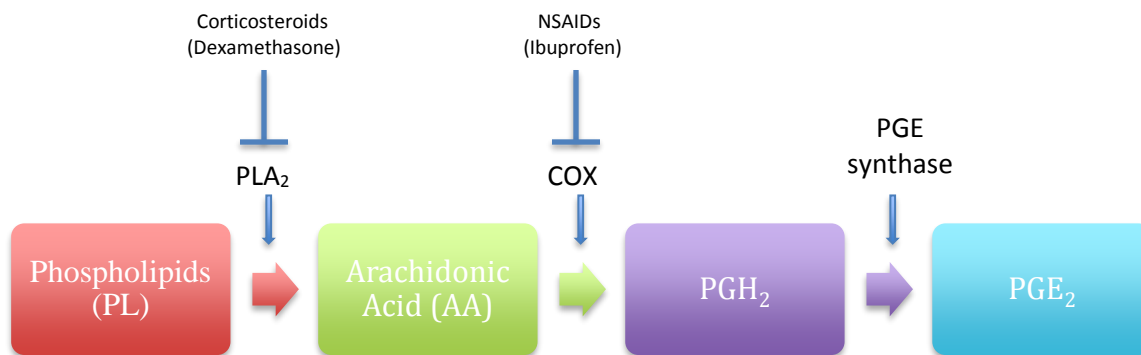
In summary, LPA may be partly responsible for the coordination of osteoblast and osteoclast activity in bone. Nucleotide signaling through the P2X7 receptors and

subsequent production of LPA reveals a new signaling axis that may play a role in skeletal mechanotransduction. In addition to the physiological roles LPA plays in regulation of osteoblast and osteoclast activity, LPA may contribute to the progression of bone diseases.

## 7 Prostaglandins

Prostaglandins (PGs) are important regulators in the inflammatory response and skeletal metabolism. PGs are lipids that stimulate G protein-coupled receptors in multiple areas of the body in an autocrine or paracrine manner. PGE<sub>2</sub> is produced in response to several factors that regulate bone metabolism, such as growth factors (PDGF), hormones (PTH) and interleukins (Flanagan and Chambers, 1992; Blackwell et al., 2010).

There are three major steps in the production of PGs such as PGE<sub>2</sub>; each are subject to regulation and each step can be rate limiting (Figure 3). First, phospholipids (PL) are metabolized to arachidonic acid at the cell membrane, through the enzymatic action of PLA<sub>2</sub>. Second is the conversion of arachidonic acid to PGH<sub>2</sub> by cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase (PTGS). COX is responsible for the formation of prostanoids, such as thromboxane and prostaglandins (Blackwell et al., 2010). Two types of COX enzymes exist: COX-1 and COX-2. The gene encoding COX-1 (*PTGS1*) is constitutively expressed and COX-1 responsible for protecting the gastrointestinal mucosa, kidney hemodynamics and platelet thrombogenesis. On the other hand, the gene encoding COX-2 (*PTGS2*) is expressed at low levels under basal conditions and is rapidly induced in response to inflammation (Rouzer and Marnett, 2009).



**Figure 3: Conversion of phospholipids to prostaglandin (PGE<sub>2</sub>).**

Membrane phospholipids (PL) are metabolized by PLA<sub>2</sub> to arachidonic acid (AA). This action is inhibited through the action of corticosteroids, such as dexamethasone. COX metabolizes AA to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>). Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, inhibit COX. Next, prostaglandin E synthase (PGE synthase) metabolizes PGH<sub>2</sub> further into PGE<sub>2</sub>, as shown above.

In bone, PGs play a role in remodeling by stimulating both resorption and formation. Application of an orthodontic force induces the production of PGs, which induces alveolar bone remodeling to promote tooth movement (Davidovitch et al., 1988; Lee, 1990; Leiker et al., 1995). Local injection of PG into the paradental tissues of rodents causes an increase in osteoclast numbers (Yamasaki et al., 1984). Reduction in the rate of orthodontic tooth movement has been shown with administration of non-specific COX inhibitors, such as indomethacin (Chumbley and Tuncay, 1986). The increase in bone resorption observed with addition of PGE<sub>2</sub> is thought to occur via an upregulation of receptor activator of NF-κB ligand (RANKL) expression and inhibition of osteoprotegerin (OPG) expression in osteoblast cells (Blackwell et al., 2010). Furthermore, PGE<sub>2</sub> is thought to stimulate cells to produce the intracellular second messenger, cAMP, which is an important regulator for bone resorption (Klein, 1970; Raisz et al., 1974). Although PGE<sub>2</sub> was initially recognized for its effects on bone

resorption, it became evident that it also stimulates bone formation. Similar to PTH, continuous administration of PGE<sub>2</sub> has shown to have resorptive effects on bone, whereas intermittent administration is anabolic (Tian et al., 2008). PGE<sub>2</sub> stimulates osteoblastic differentiation in murine marrow stromal cell cultures deficient in endogenous PG due to deletion of *Ptgs2* (Zhang et al., 2012).

Elevated PG production associated with inflammatory diseases, such as rheumatoid arthritis, can cause bone loss. Selective inhibition of COX-2 can reduce bone loss associated with inflammatory joints in rodent models (Anderson et al, 2009) and can reduce osteoclast numbers (Taketa et al., 2008).

PGs are thought to induce bone formation in response to mechanical loading and stress (Blackwell et al., 2010). Numerous studies have shown that mechanical loading increases production of COX-2 and PGs. Inhibition of COX by non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to have an inhibitory effect on bone formation in response to loading (Blackwell et al., 2010). Recent work in our lab has shown that activation of P2X7 stimulates expression of *Ptgs2* (COX-2) in osteoblasts within 3 hours of BzATP treatment (Grol et al., 2013; Na, 2016).

## 8 Rationale, Hypothesis and Objectives of the Research

Since purinergic signaling is known to be important in osteoblast differentiation and function, the aim of this project was to further investigate the signal transduction pathways that operate downstream of P2 receptor activation in cells of the osteoblast lineage.

**Objective 1: To determine if P2X7 is the receptor activated by BzATP to induce anabolic gene expression by osteoblasts.**

Previous studies by Dr. Na showed that BzATP induced anabolic gene expression by osteoblasts (Na, 2016); however, the receptor(s) mediating this effect were not identified. Considering the important role that P2X7 plays in mechanotransduction, we hypothesized that P2X7 is the receptor responsible for inducing anabolic gene expression. To test this hypothesis, we used a specific antagonist to P2X7 receptor,

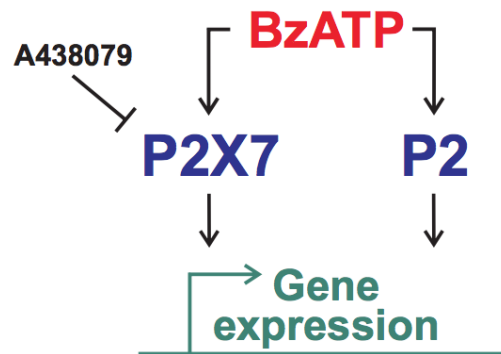
A438079, and examined its effects on BzATP-induced expression of the anabolic genes (*Ptgs2*, *Dmp1*, *c-Fos*) (Figure 4).

**Objective 2: To investigate the role of the PG and LPA signaling pathways in mediating the effects of BzATP on anabolic gene expression by osteoblasts.**

Previous studies in our lab by Panupinthu et al. showed that P2X7 activation in osteoblasts led to rapid production of the lipid mediators PGE<sub>2</sub> and LPA (Panupinthu et al., 2007; Panupinthu et al., 2008). Moreover, Dr. Na investigated the effects of dexamethasone (Na, 2016), which is known to suppress PLA<sub>2</sub> activity and therefore block production of both PGE<sub>2</sub> and LPA. Dr. Na found that dexamethasone abolished the effects of BzATP on *Ptgs2* and *Dmp1* expression, consistent with the possible involvement of PGE<sub>2</sub> and/or LPA. Therefore, we hypothesized that PGE<sub>2</sub> and/or LPA mediate the stimulatory effect of BzATP on *Ptgs2* expression.

We first assessed whether PG, LPA or both are necessary for inducing expression of *Ptgs2*. We used a COX inhibitor (that blocks PG synthesis) and/or an LPA receptor antagonist to assess the effect of loss of PG and LPA signaling on BzATP-induced gene expression (Figure 5). Next, we assessed whether PGE<sub>2</sub>, LPA or both are sufficient for inducing expression of *Ptgs2* in osteoblastic cells. To do this, we introduced PGE<sub>2</sub> and/or LPA (in the absence of exogenous nucleotides) and assessed their effect on *Ptgs2* expression (Figure 6).

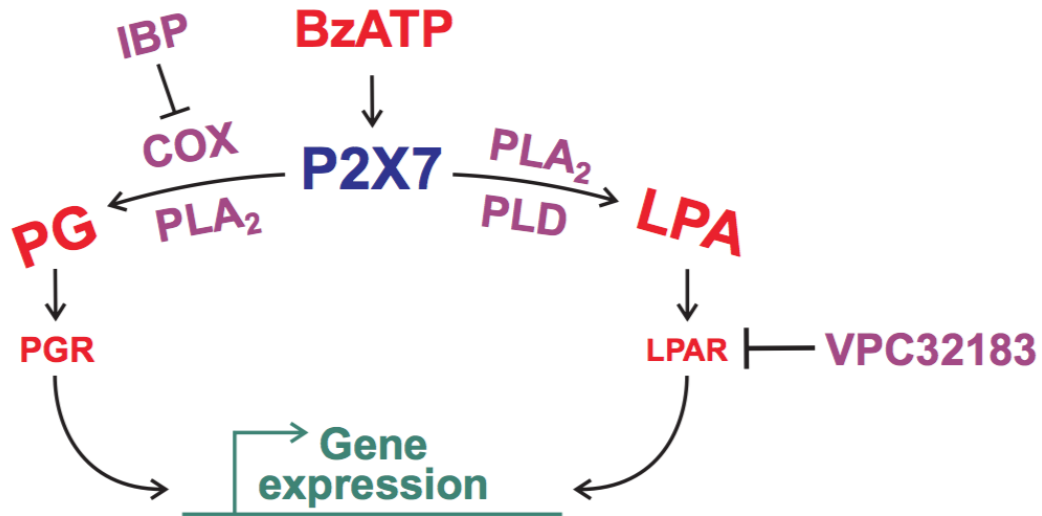
In achieving the above objectives, we hoped to gain insight on how each of these elements influences gene expression in osteoblastic cells (Figure 7).



**Figure 4: Schematic showing the potential effects of a P2X7 antagonist, A438079, on BzATP-induced gene expression at the P2X7 receptor.**

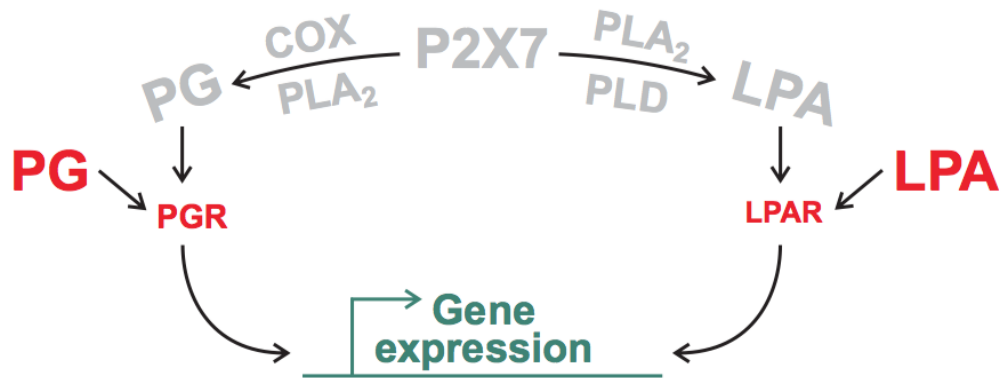
BzATP has previously been shown to induce anabolic gene expression in MC3T3-E1 cells (Na, 2016). Since BzATP is an agonist for multiple P2 receptors, it is not known if P2X7 is the receptor responsible for inducing anabolic gene expression. Using a P2X7 specific antagonist, A438079, we aimed to clarify the role of P2X7 in mediating BzATP-induced gene expression in MC3T3-E1 cells.





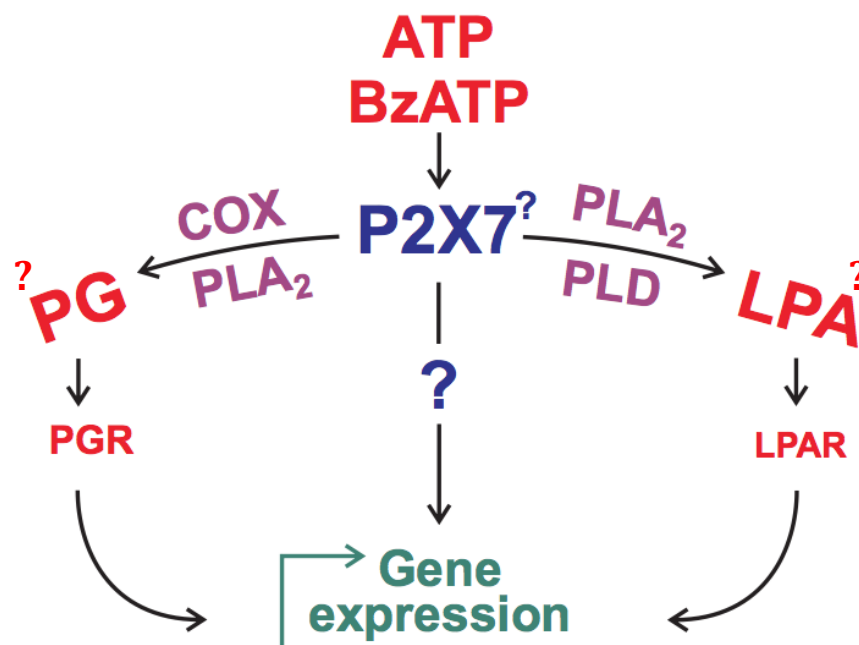
**Figure 5: Investigating the roles that PG and LPA may play downstream of P2X7 receptor activation.**

In using ibuprofen (IBP), a COX inhibitor, we examined the effects of BzATP on gene expression in the absence of PGs. Similarly, using an LPA receptor antagonist, VPC-32183, we explored the role of LPA in P2X7-driven *Ptgs2* gene expression in MC3T3-E1 cells.



**Figure 6: Effect of PG (PGE<sub>2</sub>) and LPA on gene expression in osteoblastic cells.**

By exposing MC3T3-E1 cells directly to PG, LPA or a combination of the two mediators, we assessed their individual or combined effects on *Ptgs2* gene expression. No exogenous nucleotides were added in these experiments.



**Figure 7: The proposed P2X7-PG-LPA axis and its effects on gene expression in osteoblast precursor cells.**

At least three pathways are activated by P2X7 stimulation in osteoblastic cells by endogenous ATP or exogenous BzATP. First, activation of P2X7 leads to formation of prostaglandin (PG). Second, activation of P2X7 leads to production of lysophosphatidic acid (LPA). PG and LPA then bind to their receptors (PGR and LPAR) on the same or adjacent cells to induce gene expression. Lastly, P2X7 may act through additional pathway(s) to regulate gene expression (centre arrow). Our overall objective was to explore which of these three pathways or combinations thereof, are important for anabolic gene expression in osteoblasts.

## MATERIALS AND METHODS

### 1 Materials and Solutions

$\alpha$ -Minimum essential medium ( $\alpha$ -MEM), heat-inactivated fetal bovine serum (FBS), antibiotic solution (10,000 U/ml penicillin, 10,000 mg/ml streptomycin, and 25 mg/ml amphotericin B), trypsin solution, Dulbecco's phosphate-buffered saline (DPBS) were obtained from GIBCO (Life Technologies Inc., Burlington, ON, Canada). TRIzol reagent and UltraPure distilled water (DNase/RNase-free) were obtained from Invitrogen (Life Technologies). RNeasy Mini Kit was from QIAGEN (Toronto, ON, Canada). qScript XLT One-Step RTqPCR Toughmix was purchased from Quanta. Primers and probes for *Ptgs2* (COX2, Mm00478374\_m1), *Dmp1* (Mm01208363\_m1), *c-Fos* (Mm00487425\_m1), and 18S rRNA were obtained from Applied Biosystems (Life Technologies). 2'-3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate triethylammonium salt (BzATP) and lysophosphatidic acid (LPA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ibuprofen, A438079 and PGE<sub>2</sub> were obtained from Tocris Bioscience (Bristol, UK). VPC-32183 was from Avanti Polar Lipids (Alabaster, AL, USA).

### 2 Cell Culture

A mouse calvarial pre-osteoblast cell line, MC3T3-E1 (subclone 4), was obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were cultured every 72 hours and maintained in  $\alpha$ -MEM, supplemented with 10% FBS and 1% antibiotic solution (culture medium) at 37°C and 5% CO<sub>2</sub>.

### 3 RNA Isolation

TRIZOL® reagent (Invitrogen, Paisley, UK) and RNeasy were used to extract total RNA, according to the manufacturer's instructions. Quantification of RNA was determined spectrophotometrically by measuring absorbance at 260 nm. In order to normalize RNA samples, the RNA was diluted to a final concentration of 25 ng/ $\mu$ l in RNase free water and was stored at -80 °C until amplification by Real-Time RT-PCR.

#### 4 Real-Time RT-PCR Analyses

In the first series of experiments (Objective 1), MC3T3-E1 cells were plated at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> on Falcon 6-well plates in supplemented culture medium. After 48 hours, cells were placed in serum-free medium and incubated overnight. On the day of the experiment, cells were pretreated with A438079 (10  $\mu$ M) or its vehicle (ddH<sub>2</sub>O) for 20 minutes. After 20 minutes ( $t = 0$ ), BzATP (300  $\mu$ M) or its vehicle (divalent cation free buffer, DCFB) was added and the cells were incubated for the indicated times (0, 0.5, 1 and 3 hours) (Figure 8). Total RNA was then isolated from each sample as mentioned above. Real-time PCR, using the ABI Prism 7900 HT Sequence Detector (PerkinElmer), was performed with a 15  $\mu$ L final reaction volumes containing 25 ng RNA sample, qScript XLT One-Step RTqPCR Toughmix, and one of *Ptgs2*, *c-Fos*, *Dmp1*, or 18S rRNA primers and probes. Reverse transcription was performed at 50 °C for 10 min followed by 40 cycles of amplification at an annealing temperature of 60 °C. Reactions for each sample were performed in triplicate. All samples were normalized to 18S rRNA, and time zero or vehicle-treated controls using the delta-delta cycle threshold ( $\Delta\Delta C_t$ ) method.

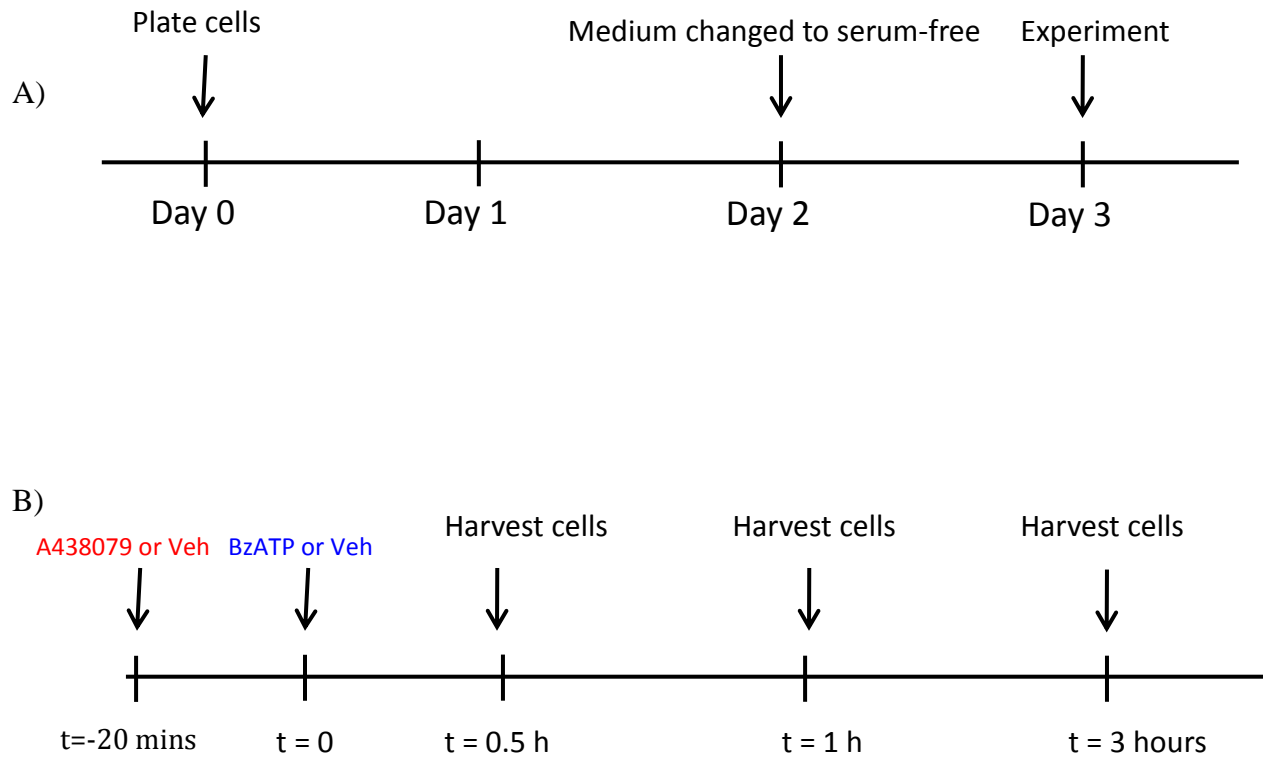
In the second series of experiments (Objective 2a), MC3T3-E1 cells were plated at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> on Falcon 6-well plates in culture medium. After 48 hours, cells were placed in serum-free medium and incubated overnight. On the day of the experiment, cells were pretreated with ibuprofen (10  $\mu$ M) or its vehicle (DMSO) and VPC-32183 (1  $\mu$ M) or its vehicle (3% BSA) for 20 minutes. After 20 minutes ( $t = 0$ ), cells were incubated with BzATP (300  $\mu$ M) or its vehicle (divalent cation-free buffer, DCFB) and total RNA was isolated as mentioned above (Figure 9). Real-time PCR was performed to assess *Ptgs2* gene expression using the techniques previously mentioned and samples were normalized using the  $\Delta\Delta C_t$  method.

In the third series of experiments (Objective 2b), MC3T3-E1 cells were plated at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> on Falcon 6-well plates in culture medium. After 48 hours, cells were placed in serum-free medium and incubated overnight. On the day of the experiment, cells were incubated with PGE<sub>2</sub> (1  $\mu$ M) or its vehicle (DMSO) and LPA (1

$\mu\text{M}$ ) or its vehicle (3% BSA) for three hours and RNA was isolated as previously described (Figure 10). Real-time PCR was completed as previously described, examining *Ptgs2* gene expression in each sample. Again, reactions for each sample were performed in triplicate. All samples were normalized to 18S rRNA, and time zero or vehicle-treated controls using the delta-delta cycle threshold ( $\Delta\Delta C_t$ ) method.

## 5 Statistical Analysis

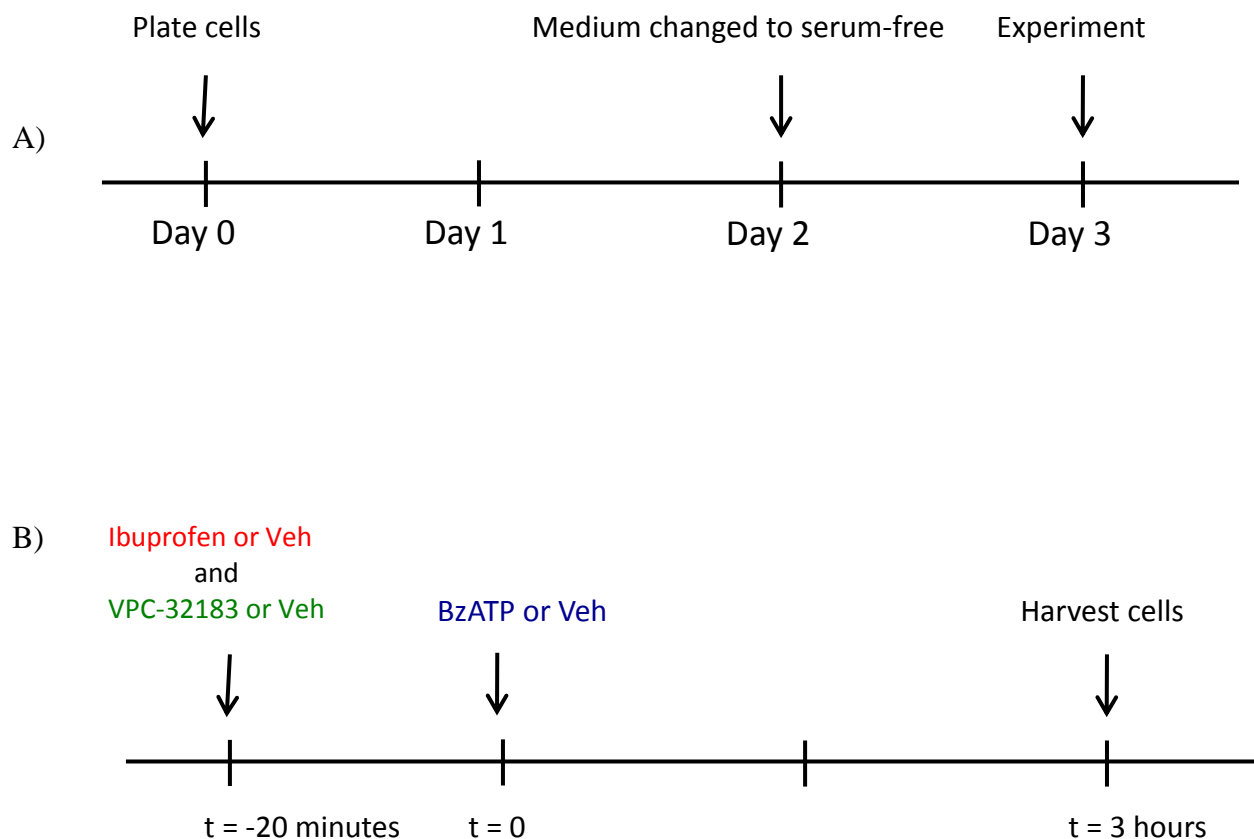
Data are shown as means  $\pm$  standard error of the mean (SEM) for the number ( $n$ ) of experiments indicated, each performed in triplicate. Differences among three or more groups were evaluated by one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* multiple comparisons test. Differences were accepted as statistically significant at  $p < 0.05$ . Data analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).



**Figure 8. Timeline of the first series of experiments.**

A) MC3T3-E1 cells were plated at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> in Falcon 6-well plates in culture medium (day 0). After 2 days, cells were placed in serum-free medium.

B) On the day of the experiment (day 3), cells were pretreated with A438079 (10  $\mu$ M) or its vehicle (ddH<sub>2</sub>O) for 20 minutes. After 20 minutes (t = 0), BzATP (300  $\mu$ M) or its vehicle (DCFB) was added. Total RNA was then isolated using Trizol at 0, 0.5, 1, and 3 hours.

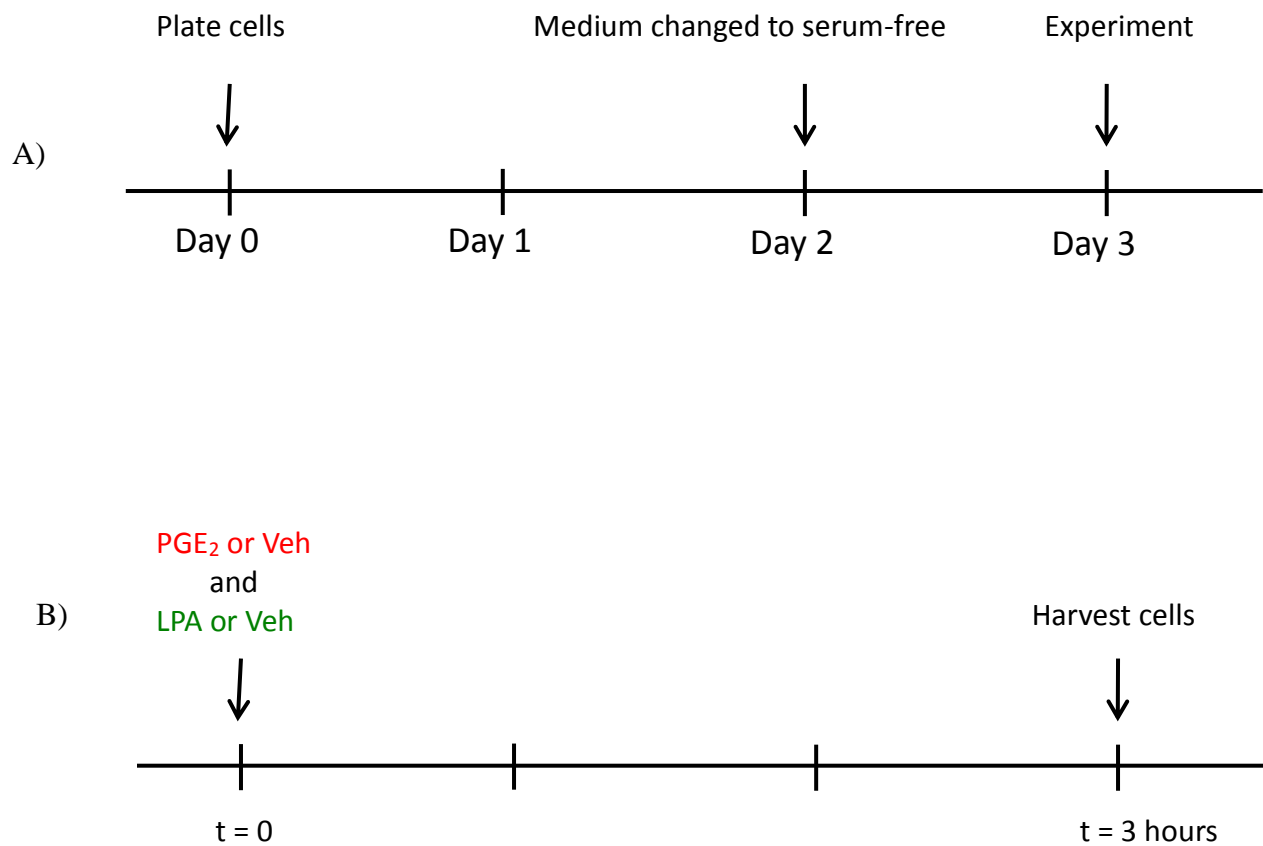


**Figure 9. Timeline of the second series of experiments.**

A) MC3T3-E1 cells were plated at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> in Falcon 6-well plates in culture medium (day 0). After 2 days, cells were placed in serum-free medium.

B) On the day of the experiment (day 3), cells were incubated with ibuprofen (10  $\mu$ M) or its vehicle (DMSO) and VPC-32183 (1  $\mu$ M) or its vehicle (3% BSA) for 20 minutes. At time 0, BzATP (300  $\mu$ M) or its vehicle (DCFB) was added in the continued presence of ibuprofen, VPC-32183 or vehicle. Total RNA was then isolated using Trizol at 3 hours.





**Figure 10. Timeline of the third series of experiments.**

A) MC3T3-E1 cells were plated at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup> in Falcon 6-well plates in culture medium (day 0). After 2 days, cells were placed in serum-free medium.

B) On the day of the experiment (day 3), cells were incubated with PGE<sub>2</sub> (1  $\mu$ M) or its vehicle (DMSO) and LPA (1  $\mu$ M) or its vehicle (3% BSA) for 3 hours. Total RNA was then isolated using Trizol at 3 hours.

## RESULTS

1 BzATP-induced stimulation of *Ptgs2* (COX-2) expression was inhibited by the P2X7 antagonist, A438079

*Ptgs2* encodes prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase-2 (COX-2), which mediates the synthesis of PGs (Blackwell et al., 2010).

Changes in *Ptgs2* expression were examined in MC3T3-E1 cells following treatment with vehicle (DCF Buffer) or BzATP (300  $\mu$ M) in the presence or absence of the specific P2X7 antagonist, A438079 (10  $\mu$ M). Similar to results previously shown in our lab (Grol et al., 2013), BzATP induced peak *Ptgs2* expression at three hours ( $342 \pm 52$  fold increase relative to vehicle-treated cultures at time 0) (Figure 11, blue). Significant *Ptgs2* expression ( $320 \pm 47$  fold) was also observed at one hour, a similar time point to previous studies done in our lab (Na, 2016). BzATP had a significant effect on *Ptgs2* gene expression at both 1 and 3 h ( $p < 0.05$ ). A438079 alone did not significantly affect *Ptgs2* expression; however, A438079 significantly blocked BzATP-induced *Ptgs2* expression at 1 h and 3 h ( $p < 0.05$ ) (Figure 11, green). Taken together, these data indicate that P2X7 is the receptor responsible for BzATP-induced *Ptgs2* expression in MC3T3-E1 cells.

2 BzATP-induced stimulation of *Dmp1* expression was inhibited by A438079

*Dmp1* encodes dentin matrix acidic phosphoprotein 1, which is present bone and dentin, and has been shown to be necessary for their mineralization (George et al., 1993; MacDougall et al., 1998; Feng et al., 2006). Bone cells, including osteocytes and osteoblasts, express *Dmp1* (Feng et al., 2003; Toyosawa et al., 2012). BzATP stimulation of *Dmp1* expression, which was greatest at 3 h ( $6070 \pm 2014$  fold increase), was completely inhibited by treatment with A438079 ( $p < 0.05$ ) (Figure 12, compare blue and green lines). Maximal expression of *Dmp1* at 3 hours was consistent with previous findings in our lab (Na, 2016).

### 3 BzATP-induced stimulation of *c-Fos* expression was inhibited by A438079

*c-Fos* is an immediate early gene, which has been implicated in osteoblast proliferation, differentiation and survival (Dixon and Sims, 2000). In osteoblast precursor cells, FOS protein expression is high during proliferation, but levels decline during further maturation and differentiation (McCabe et al., 1995).

Significant *c-Fos* expression was seen at 30 minutes ( $63 \pm 21$  fold) and one hour ( $67 \pm 18$ ) following BzATP stimulation (Figure 13, blue). Expression was transient, returning to a basal level by 3 hours. Similar results were seen previously in our lab with peak *c-Fos* expression seen at 30 minutes (Na, 2016). Again, treatment with A438079 completely inhibited *c-Fos* expression induced by BzATP (Figure 13, green) ( $p < 0.05$ ). Thus, the effects of BzATP on expression of *Ptgs2*, *Dmp1* and *c-Fos* appear to be mediated by P2X7.

### 4 BzATP-induced stimulation of *Ptgs2* expression was inhibited by treatment with VPC-32183 and ibuprofen

For Objective 2, we chose to focus our studies on *Ptgs2* expression for several reasons. First, COX-2 inhibitors have received an abundance of attention in recent years with respect to their effects on bone healing and remodeling, and their possible actions on orthodontic tooth movement (Cottrell and O-Connor, 2010; Fang et al, 2016; Proffit, 2012). Additionally, we had previously observed dramatic stimulation of *Ptgs2* in response to BzATP stimulation in osteoblastic cells (Grol et al., 2013; Na, 2016).

Ibuprofen is a non-selective inhibitor for COX. At the 3 h time point, ibuprofen significantly reduced BzATP-induced expression of *Ptgs2* ( $p < 0.05$ ) (Figure 14).

VPC-32183 is a selective antagonist of LPA receptors LPA<sub>1</sub> and LPA<sub>3</sub> (Heasley, 2004). In MC3T3-E1 cells, the LPA<sub>1</sub> receptor is the most abundant receptor for LPA, with LPA<sub>3</sub> expression virtually undetectable (Masiello et al., 2006). When MC3T3-E1 cells

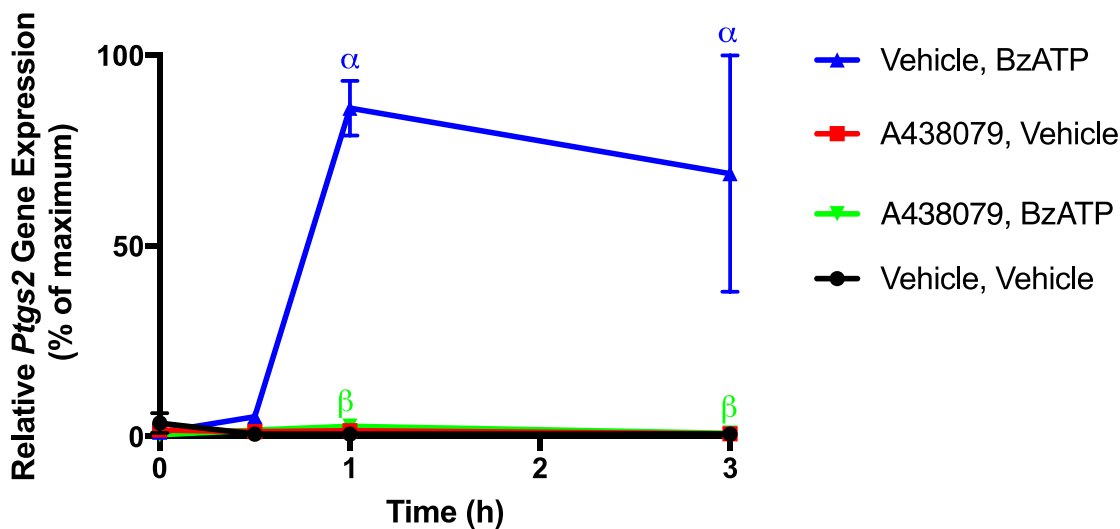
were harvested at 3 h, VPC-32183 significantly reduced BzATP induced expression of *Ptgs2* ( $p < 0.05$ ) (Figure 14).

The combined effect of VPC-32183 and ibuprofen on MC3T3-E1 cells at 3 hours was virtually complete inhibition of *Ptgs2* expression ( $p < 0.05$ ) (Figure 14). Thus, both PG and LPA signaling appear to be necessary for mediating the effects of P2X7 activation on *Ptgs2* expression.

5 PGE<sub>2</sub> and LPA had a synergistic stimulatory effect on *Ptgs2* gene expression in MC3T3-E1 cells

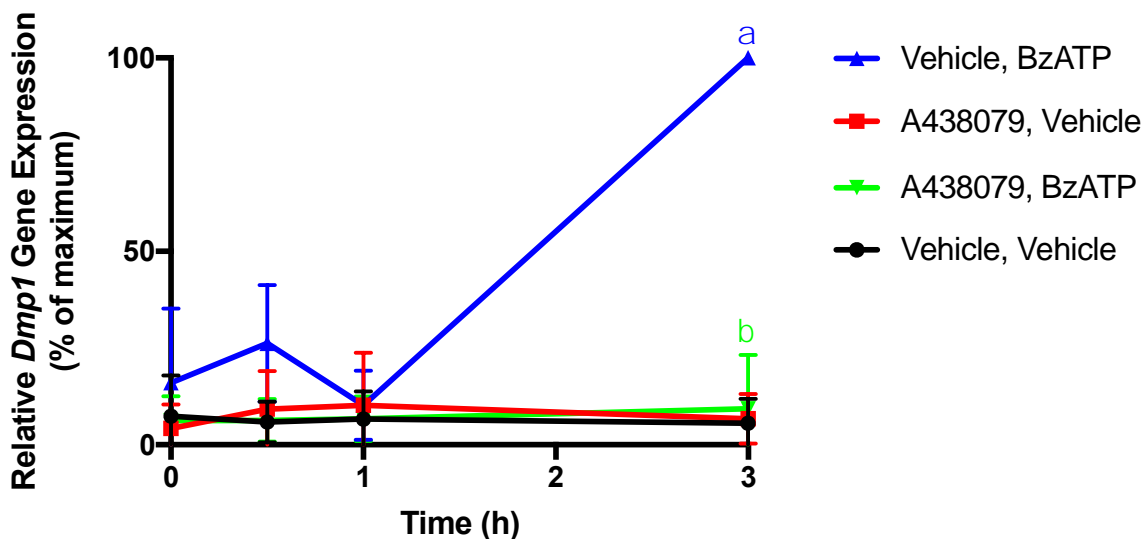
Previously published dose-response data for both PGE<sub>2</sub> (Suda et al., 1998) and LPA (Nochi et al., 2008) led us to select 1 μM concentrations for both factors, as this concentration resulted in maximum anabolic gene expression in osteoblastic cells. Interestingly, in our experiments, PGE<sub>2</sub> or LPA alone did not significantly stimulate *Ptgs2* gene expression in MC3T3-E1 cells. However, their combined effect was a significant increase in *Ptgs2* expression after 3 h of incubation ( $739 \pm 500$  fold compared to vehicle-treated cultures,  $p < 0.05$ , Figure 15). Thus, in combination, PGE<sub>2</sub> and LPA appear to be sufficient to mimic the effect of P2X7 activation on *Ptgs2* expression.

NB: Please refer to Appendix A for data presented as relative gene expression without normalization as percentage of the maximum value.



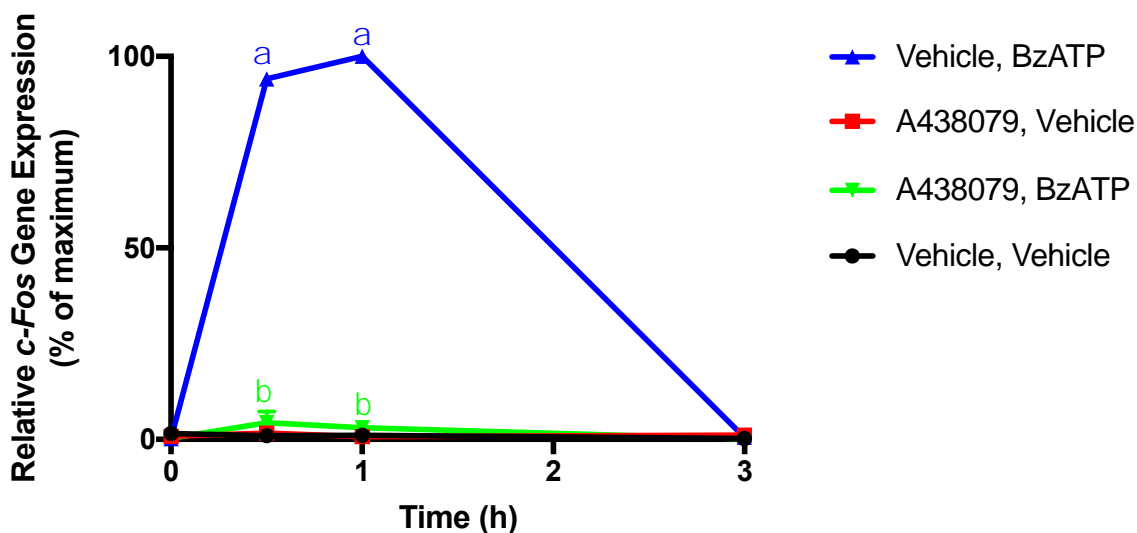
**Figure 11: BzATP-induced stimulation of *Ptgs2* expression was completely inhibited by the P2X7 antagonist A438079.**

MC3T3-E1 cells were pretreated with A438079 (10  $\mu$ M) or its vehicle for 20 minutes and then incubated with BzATP (300  $\mu$ M) or its vehicle at time 0 (in the continued presence of A438079 or its vehicle). Total RNA was isolated at the indicated times. Real-time RT-PCR was performed to assess expression of *Ptgs2*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for vehicle-treated cultures at time 0. Data are shown as percentages of the maximum value in each individual experiment and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). The mean maximum value (100%) for these 3 experiments was  $342 \pm 52$  fold increase relative to vehicle-treated cultures at time 0. Data were analyzed by two-way ANOVA followed by a Bonferroni *post hoc* multiple comparisons test.  $\alpha$  indicates a significant difference between BzATP and vehicle at each time point;  $\beta$  indicates a significant effect of the P2X7 antagonist, A438079;  $p < 0.05$ .



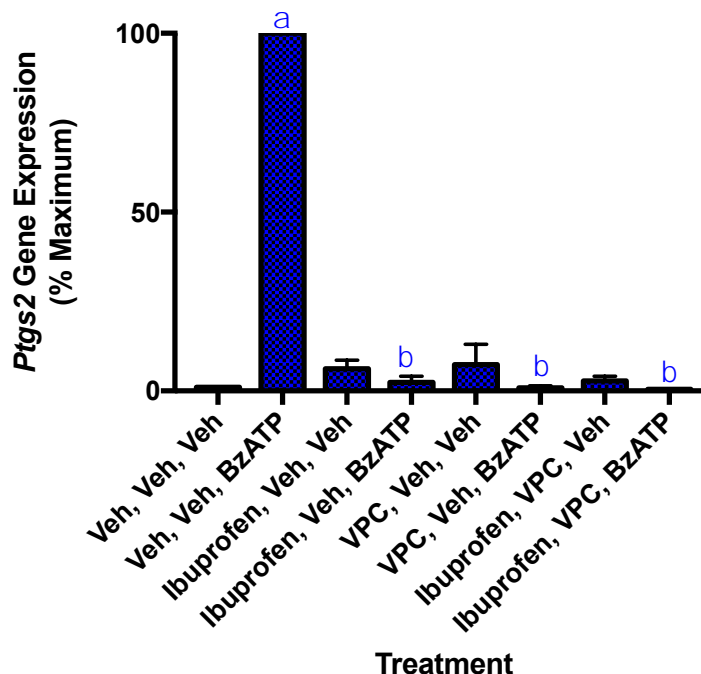
**Figure 12: BzATP-induced stimulation of *Dmp1* expression was completely inhibited by A438079.**

MC3T3-E1 cells were pretreated with A438079 (10  $\mu$ M) or its vehicle for 20 minutes and then incubated with BzATP (300  $\mu$ M) or its vehicle at time 0 (in the continued presence of A438079 or its vehicle). Total RNA was isolated at the indicated times. Real-time RT-PCR was performed to assess expression levels of *Dmp1*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for vehicle-treated cultures at time 0. Data are shown as percentages of the maximum value in each individual experiment and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). The mean maximum value (100%) for these 3 experiments was  $6070 \pm 2014$  fold increase relative to vehicle-treated cultures at time 0. Differences were evaluated by two-way ANOVA followed by a Bonferroni multiple comparisons test.  $\alpha$  indicates a significant difference between BzATP and vehicle at each time point;  $\beta$  indicates a significant effect of the P2X7 antagonist, A438079;  $p < 0.05$ .



**Figure 13: BzATP-induced stimulation of *c-Fos* expression was completely inhibited by A438079.**

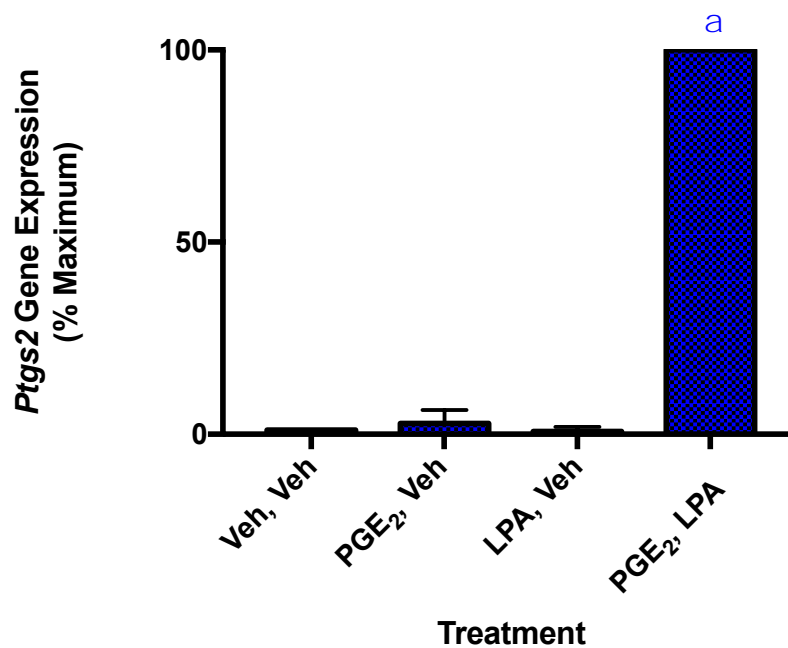
MC3T3-E1 cells were pretreated with A438079 (10  $\mu$ M) or its vehicle for 20 minutes and then incubated with BzATP (300  $\mu$ M) or its vehicle at time 0 (in the continued presence of A438079 or its vehicle). Total RNA was isolated at the indicated times. Real-time RT-PCR was performed to assess expression levels of *c-Fos*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for vehicle-treated cultures at time 0. Data are shown as percentages of the maximum value in each individual experiment and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). The mean maximum value (100%) for these 3 experiments was  $67 \pm 18$  fold increase relative to vehicle-treated cultures at time 0. Differences were evaluated by two-way ANOVA followed by a Bonferroni multiple comparisons test.  $\alpha$  indicates a significant difference between BzATP and vehicle at each time point;  $\beta$  indicates a significant effect of the P2X7 antagonist, A438079;  $p < 0.05$ .



**Figure 14: BzATP-induced stimulation of *Ptgs2* expression was inhibited by the COX inhibitor ibuprofen and the LPA receptor antagonist VPC-32183.**

MC3T3-E1 cells were pretreated with ibuprofen (10  $\mu$ M) or its vehicle (DMSO) and VPC-32183 (VPC) (1  $\mu$ M) or its vehicle (3% BSA) for 20 minutes. After 20 minutes, BzATP (300  $\mu$ M) or its vehicle (DCFB) was added (in the continued presence of ibuprofen, VPC-32183, or vehicle). Total RNA was isolated at 3 hours for all treatment groups. Real-time RT-PCR was performed to assess the expression of *Ptgs2*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for cultures treated only with vehicles. Data are shown as percentages of the maximum value in each individual experiment and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). The mean maximum value (100%) for these 3 experiments was  $59 \pm 7$  fold increase relative to vehicle-treated cultures. Differences were evaluated by two-way ANOVA followed by a Bonferroni multiple comparisons test.  $\alpha$  indicates a significant difference between BzATP and vehicle at each time point;  $\beta$  indicates a significant effect of ibuprofen and/or VPC-32183;  $p < 0.05$ .





**Figure 15: PGE<sub>2</sub> and LPA combined induced *Ptgs2* gene expression in MC3T3-E1 cells.**

MC3T3-E1 cells were incubated with PGE<sub>2</sub> (1  $\mu$ M) or its vehicle (DMSO) and LPA (1  $\mu$ M) or its vehicle (3% BSA) at time 0. No exogenous BzATP was added. Total RNA was isolated at 3 hours for all treatment groups. Real-time RT-PCR was performed to assess expression levels of *Ptgs2*. Data were normalized to levels of 18S ribosomal RNA, and relative to values for vehicle-treated cultures. Data are shown as percentages of the maximum value in each individual experiment and are presented as means  $\pm$  S.E.M. ( $n = 3$  independent experiments, each performed in triplicate). The mean maximum value (100%) for these 3 experiments was  $739 \pm 500$  fold increase relative to vehicle-treated cultures at time 0. Differences were evaluated by one-way ANOVA followed by a Bonferroni multiple comparisons test.  $\alpha$  indicates a significant difference between PGE<sub>2</sub>, LPA or both and vehicle;  $p < 0.05$ .

## DISCUSSION

## 1 Summary and Conclusions

*Objective 1: To determine if P2X7 is the receptor activated by BzATP to induce anabolic gene expression by osteoblasts.*

- As shown previously by Dr. Na, BzATP stimulated expression of *Ptgs2*, *Dmp1* and *c-Fos*. We found that the specific P2X7 antagonist, A438079, inhibited expression of all three genes. These data indicate that P2X7 is the receptor activated by BzATP to induce expression of these genes in MC3T3-E1 osteoblastic cells.

*Objective 2: To investigate the role of the PG and LPA signaling pathways in mediating the effects of BzATP on anabolic gene expression by osteoblasts.*

- BzATP-induced stimulation of *Ptgs2* expression was reduced by treatment with the LPA<sub>1/3</sub> receptor antagonist VPC-32183.
- BzATP-induced stimulation of *Ptgs2* expression was reduced by treatment with the COX inhibitor ibuprofen.
- VPC-32183 and ibuprofen combined abolished *Ptgs2* gene expression in BzATP-stimulated MC3T3-E1 cells.
- Individually, PGE<sub>2</sub> or LPA had little effect on *Ptgs2* gene expression in MC3T3-E1 cells.
- Combined, PGE<sub>2</sub> and LPA displayed a dramatic synergistic effect on *Ptgs2* gene expression.
- Taken together, LPA and PG signaling pathways appear to be both necessary and sufficient to mediate the effect of P2X7 on *Ptgs2* expression in MC3T3-E1 osteoblastic cells.

## 2 P2X7 regulates expression of anabolic genes

In Objective 1, we aimed to determine if P2X7 is the receptor activated by BzATP that induces anabolic gene expression in osteoblasts. BzATP is not a specific agonist for the P2X7 receptor. BzATP has previously been shown to activate a number of other receptors including P2Y11, P2X1, and P2X4 with similar potency (Bianchi et al., 1999; Communi et al., 1999; Naemsch et al., 1999; North, 2002). Using a specific P2X7 antagonist, we were able to show that BzATP stimulates expression of *Ptgs2*, *Dmp1* and *c-Fos* by activating P2X7.

Previously, Dr. Na examined changes in *Ptgs2*, *Dmp1* and *c-Fos* expression following BzATP activation of P2X7 in osteoblastic cells (Na, 2016). Furthermore, Dr. Na showed that dexamethasone has an inhibitory effect on BzATP-induced expression of *Ptgs2* and *Dmp1*. Interestingly, Dr. Na found that BzATP-induced expression of *c-Fos* was not affected by treatment with dexamethasone, indicating the potential for specificity in dexamethasone action.

*Ptgs2* encodes prostaglandin-endoperoxide synthase-2 (COX-2) and is important for PG production. PGs are imperative in regulation of bone metabolism, by inducing both bone formation and resorption, and they are necessary in fracture healing (Blackwell et al., 2010). Previous studies have shown that osteoblast mechanotransduction may involve the production of prostaglandins, such as PGE<sub>2</sub> (Li et al., 2005). With this in mind, we examined changes in *Ptgs2* expression following application of BzATP with and without the addition of the P2X7 selective inhibitor, A438079. A438079 is a recently developed antagonist that has been shown to specifically block P2X7 (Nelsen et al., 2006; Donnelly-Roberts and Jarvis, 2007). We confirmed that BzATP activation of P2X7 leads to significant increases in *Ptgs2* expression at similar time points as previously observed in our past studies (Grol et al., 2013; Na, 2016). Furthermore, we showed that BzATP-induced expression of *Ptgs2* is inhibited by A438079 and therefore mediated through activation of P2X7.

*Dmp1* encodes dentin matrix acidic phosphoprotein 1, which is found in bone, dentin and cementum (George et al., 1993). *Dmp1* is expressed by both osteoblasts and osteocytes and is critical in bone mineralization. In this study, we saw significant *Dmp1* expression at 3 hours as observed in our previous studies (Na, 2016). Again, A438079 completely inhibited *Dmp1* expression, implicating P2X7 in this effect.

*c-Fos* is an immediate early gene, which encodes a transcription factor that plays a critical role in regulating differentiation and proliferation of bone and cartilage cells (Hipskind and Bilbe, 1998). Overexpression of *c-Fos* has been found to be associated with the development of osteosarcomas and chondrosarcomas in mice, whereas failure to express *c-Fos* leads to lack of osteoclasts and development of osteopetrosis in mice (David et al., 2005; Wang et al., 1992). These findings suggest that *c-Fos* plays an important role in the regulation of bone cells. In the present study, we confirmed Dr. Na's previous observation that BzATP leads to an increase of *c-Fos* expression at 30 minutes (Na, 2016). Again, A438079 completely abolished *c-Fos* expression implicating P2X7.

Thus, we have shown that in osteoblastic cells activation P2X7 stimulates expression of *Ptgs2*, *Dmp1* and *c-Fos*, which should lead to anabolic effects in bone. Our findings are consistent with evidence from others implicating P2X7 in osteogenesis, including reduced alkaline phosphatase activity in osteoblasts from P2X7 KO mice (Panupinthu et al., 2008), decreased periosteal bone formation in P2X7 KO mice (Ke et al., 2003), and their attenuated anabolic response to mechanical loading (Li et al., 2005). Furthermore, an increased risk of osteoporosis in humans is associated with several polymorphisms in P2X7 linked to impaired receptor function (Gartland et al., 2012; Jorgensen et al., 2012; Ohlendorff et al., 2007). Our findings provide further evidence of a role for P2X7 in stimulating bone formation.

### 3 Role of lipid signaling pathways in mediating effects of P2X7

Lysophosphatidic acid (LPA) is a lipid mediator that interacts with G protein-coupled receptors and has been shown to be involved with osteoblast differentiation and

chemotaxis (Blackburn and Mansell, 2011; Sims et al., 2013; Sheng et al., 2015). There are several receptors for LPA; LPA<sub>1</sub> is the predominant receptor for LPA on osteoblastic cells (Masiello et al., 2006). Several studies have shown that LPA<sub>1</sub> plays a role in bone formation. For example, Gennero et al. found that LPA<sub>1</sub> KO mice displayed significant bone defects and low bone mass, indicating that LPA<sub>1</sub> may play an important role in osteogenesis (Gennero et al., 2011). Another study found that inhibition of LPA<sub>1</sub> in human mesenchymal stem cells decreased LPA-induced rise in intracellular Ca<sup>2+</sup> and cAMP signaling as well as subsequent osteogenesis (Liu et al., 2010). LPA-treated MC3T3-E1 cells show alterations in their cytoskeleton and elevations in Ca<sup>2+</sup>, effects that resemble the osteoblastic cells' response to fluid shear (Waters et al., 2007). Waters et al. found that LPA-treated MC3T3-E1 cells expressed genes necessary for skeletal repair, including several inflammatory mediators (Waters et al., 2007).

Prostaglandins (PGs) are important in inflammation and are instrumental in mediating the effects of mechanical stress (Blackwell et al., 2010). In orthodontics, PGs have been found to stimulate both osteoclastic bone resorption and osteoblastic bone formation (Davidovitch et al., 1988; Yamasaki et al., 1984). PGE<sub>2</sub> has four receptor subtypes that it binds to: EP1-EP4 (Funk, 2001). MC3T3-E1 cells express both EP1 and EP4 receptor subtypes (Suda et al., 1998). The stimulatory effects of PGE<sub>2</sub> on osteoblast differentiation and subsequent bone formation are thought to be mediated by the EP4 receptor (Yoshida et al., 2002).

Previous results from our lab and others show that P2X7 activation is linked to the production of lipid mediators, which may have osteogenic effects in bone. Li et al. showed that response to mechanical loading and PGE<sub>2</sub> release was impaired in P2X7 receptor deficient mice (Li et al., 2005). In our lab, Panupinthu et al. showed that signaling through P2X7 induces production of LPA and PGE<sub>2</sub>, and enhances osteogenesis (Panupinthu et al., 2007; Panupinthu et al., 2008). Panupinthu et al. found that the LPA<sub>1/3</sub> antagonist VPC-32183 abolished the effect of BzATP on mineral deposition in rat calvarial cell cultures. Furthermore, Panupinthu et al. used ibuprofen to block the effects of BzATP on PG synthesis. Similar to the actions of VPC-32183, ibuprofen abolished the effects of BzATP on mineralization (Panupinthu et al., 2008).

We examined the role of PGs and LPA in BzATP-induced *Ptgs2* expression by inhibiting PGE<sub>2</sub> production and/or blocking the LPA receptor. In treating MC3T3-E1 cells with ibuprofen, an inhibition of *Ptgs2* expression was seen. VPC-32183 also had an inhibitory effect on BzATP-induced *Ptgs2* expression. In combination, ibuprofen and VPC-32183 completely abolished *Ptgs2* expression.

Lastly, we examined the effects of PGE<sub>2</sub> and LPA on *Ptgs2* expression in MC3T3-E1 cells. We found that the addition of either lipid mediator individually did not have a significant effect on *Ptgs2* expression; however, the combined addition of PGE<sub>2</sub> and LPA significantly increased in *Ptgs2* expression. We show for the first time that PGE<sub>2</sub> and LPA have a synergistic effect that simulates BzATP-induced P2X7 activation. This finding indicates that both lipid mediators are necessary and sufficient in combination to mimic BzATP-induced P2X7 activation. Thus, they may play important roles in P2X7-mediated osteogenesis and mechanotransduction.

#### 4 Limitations of the study and suggestions for future studies

There are a few limitations of this study as outlined below.

- a) Our study was limited to investigation of specific genes, but this was necessary as a preliminary step to determine the types of genes being expressed in response to various treatments administered. In addition, time constraints precluded us from examining protein levels and *in vitro* mineralization.
- b) In using a mouse osteoblast precursor cells line, we limit translation of our results to human osteoblasts, as there are inherent species-specific differences between mouse and human osteoblasts.
- c) While our study was *in vitro* in nature, it provides a useful model for understanding the effects of P2X7 receptor activation. These results may be translatable to *in vivo* effects, but future studies will be needed to determine this.

A number of future studies would be useful to address these limitations and answer questions that have arisen as a result of this study. These include:

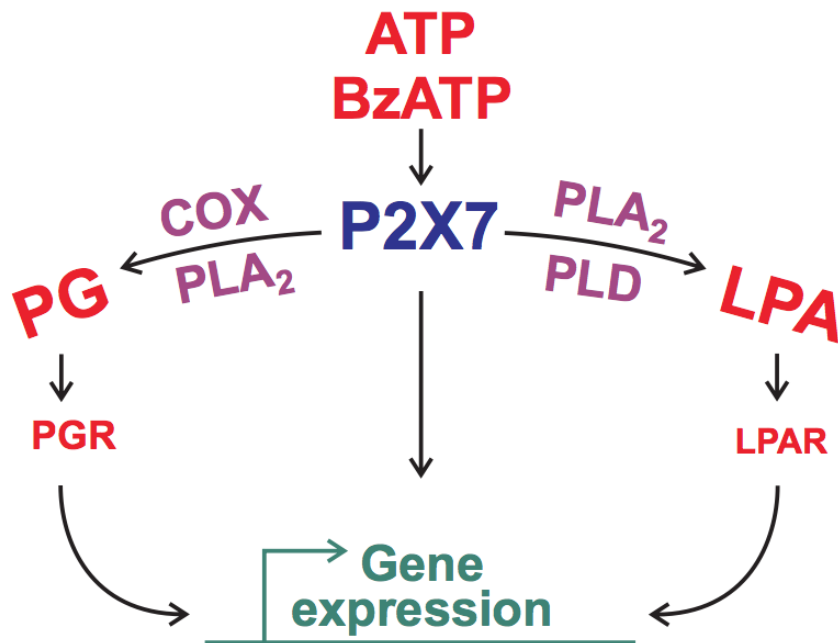
- a) Other genes, including genes not regulated by dexamethasone (e.g. *c-Fos*), which we would expect are regulated by pathways downstream of P2X7 other than PG and LPA pathways.
- b) Examine protein levels with a similar experimental design.
- c) We expect that dexamethasone should inhibit P2X7-activated production of LPA and PGE<sub>2</sub>. Future studies should directly measure the effects of dexamethasone on the release of these lipid mediators.
- d) Determine how PG and LPA interact synergistically. This would include identifying: which LPA and PG receptors are involved, which signaling pathways are activated downstream of the receptors, and how these pathways interact to stimulate *Ptgs2* expression.
- e) A study done in a primary osteoblast cultures looking at an extended timeline post BzATP treatment should clarify whether the expression of anabolic genes in osteoblasts translates to increased matrix mineralization.
- f) Others have shown that PG inhibitors block mechanically induced bone formation in animal models (Cottrell and O-Connor, 2010). It would be interesting to determine whether dexamethasone or LPA<sub>1/3</sub> receptor antagonists have similar effects. Similarly, it would be interesting to assess the effects of these agents on orthodontic tooth movement in an animal model.

## 5 Relevance of the study in orthodontic tooth movement

Although the cellular mechanisms underlying orthodontic tooth movement (OTM) have been clearly outlined, the precise pathways mediating mechanotransduction remain to be elucidated. In our study, we aimed to establish the role of P2X7 in anabolic gene expression in osteoblastic cells. In expanding our basic science knowledge and understanding molecular mechanisms involved in OTM, we may design more physiologic and efficient methods to achieve better orthodontic results. Any mechanism involved with inhibiting or delaying the events contributing to mechanical stimulation and subsequent bone remodeling may have negative consequences to OTM. Here we show that the non-selective COX inhibitor ibuprofen has a negative effect on osteoblast activation and potentially bone formation via its inhibition of *Ptgs2* expression. This

finding may assist in explaining why other studies have found that COX inhibitors, such as indomethacin, have an inhibitory effect on OTM (Chumbley and Tuncay, 1986). Since OTM is dependent upon the coordinated activity of osteoblasts and osteoclasts, disruption in the activity of one of these cell types may inhibit efficient tooth movement. Further implicating NSAIDs in altered bone remodeling, numerous studies have indicated that NSAIDs may impair bone healing and repair (Cottrell and O-Connor, 2010). This is believed to occur due to inhibition of prostaglandin production, which reduces osteoclast and osteoblast activation necessary for bone healing. Although evidence exists to suggest that low dose and short-term use of NSAIDs may not have a significant effect on the rate of OTM clinically (Proffit, 2012; Fang et al., 2016), further studies are needed to clarify future recommendations.





**Figure 16: Summary of the findings.**

In conclusion, we show that P2X7 is the receptor activated by BzATP to induce anabolic gene expression in osteoblasts. Genes such as *Ptgs2*, *Dmp1* and *c-Fos* are up regulated through BzATP stimulation and are abolished through inhibition with a P2X7-specific antagonist, A438079. Furthermore, we show that a P2X7-LPA-PGE<sub>2</sub> axis may have a regulatory role in osteogenesis.

## REFERENCES

- Anderson GD, Keys KL, De Ciechi PA, Masferrer JL (2009). Combination therapies that inhibit cyclooxygenase-2 and leukotriene synthesis prevent disease in murine collagen induced arthritis. *Inflamm Res* 58:109-117.
- Baron R (2003). *General Principles of Bone Biology* Washington, DC.: American Society for Bone and Mineral Research.
- Bianchi BR, Lynch KL, Touma E, Niforatos W, Burgard EC, Alexander KM, Park HS, Yu H, Metzger R, Kowaluk E, Jarvis MF, van Biesen T (1999). Pharmacological characterization of recombinant human and rat P2X receptor subtypes. *Eur J of Pharm* 376(1):127-138.
- Blackburn J, Mansell JP (2012). The emerging role of lysophosphatidic acid (LPA) in skeletal biology. *Bone* 50:756-762.
- Blackwell KA, Raisz LG, Pilbeam CC (2010). Prostaglandins in bone: bad cop, good cop? *Trends in endocrinology and metabolism: TEM* 21(5):294-301.
- Bodin P, Burnstock G. Increased release of ATP from endothelial cells during acute inflammation (1998). *Inflamm Res*;47:351-354.
- Bonewald LF (1999). Establishment and characterization of an osteocyte-like cell line, MLO-Y4. *Journal of bone and mineral metabolism* 17(1):61-65.
- Boyce BF, Xing L (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Archives of biochemistry and biophysics* 473(2):139-146.
- Boyle WJ, Simonet WS, Lacey DL (2003). Osteoclast differentiation and activation. *Nature* 423(6937):337-342.
- Brough D, Le Feuvre RA, Wheeler RD, Solovyova N, Hilfiker S, Rothwell NF (2003). Ca<sup>+2</sup> stores and Ca<sup>+2</sup> entry differentially contribute to the release of IL-1 beta and IL-1 alpha from murine macrophages. *J Immunol*;170:3029-3036.
- Buckley KA, Golding SL, Rice JM, Dillon JP, Gallagher JA (2003). Release and interconversion of P2 receptor antagonists by human osteoblast-like cells. *FASEB J*. 17:1401-1410.
- Buckley KA, Golding SL, Rice JM, Dillon JP, Gallagher JA (2003). Release and interconversion of P2 receptor agonists by human osteoblast-like cells. *FASEB journal* : 17(11):1401-1410.
- Burnstock G (1976). Purinergic receptors. *Journal of theoretical biology* 62(2):491-503.

Burnstock G, Kennedy C (1985). Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharm*; 16(5):433-440.

Burnstock G (2004). Introduction: P2 receptors. *Current topics in medicinal chemistry* 4(8):793-803.

Burnstock G (2007). Purine and pyrimidine receptors. *Cellular and molecular life sciences : CMLS* 64(12):1471-1483.

Chen L, Brosnan CF. Regulation of immune response by P2X7 receptor (2006). *Crit Rev Immunol*;26:499-513.

Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J (2005). Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory pain and neuropathic pain. *Pain*; 114(3):386-396.

Chumbley AB, Tuncay OC (1986). The effects of indomethacin on the rate of tooth movement in cats. *Am J Orthod* 89:312-314.

Clarke B (2008). Normal bone anatomy and physiology. *Clinical journal of the American Society of Nephrology : CJASN* 3 Suppl 3; S131-139.

Communi D, Robaye B, Boeynaems JM (1999). Pharmacological characterization of the human P2Y11 receptor. *British J of Pharm* 128(6):1199-1206.

Cottrell J, O'Connor P (2010). Effect of Non-Steroidal Anti-Inflammatory Drugs on Bone Healing. *Pharmaceuticals* 3:1668-1693.

David JP, Mehic D, Bakiri L, Schilling AF, Mandric V, Priemel M, Idarraga MH, Reschke MO, Hoffman O, Amling M, Wagner EF (2005). Essential role of RSK2 in c-Fos-dependent osteosarcoma development. *J Clin Invest* 115(3):664-672.

Davidovitch Z, Nicolay OF, Ngan PW, Shanfeld JL (1988). Neurotransmitters, cytokines, and the control of alveolar bone remodeling in orthodontics. *Dental clinics of North America* 32(3):411-435.

Dixon S, Sims S (2000). P2 Purinergic receptors on osteoblasts and osteoclasts: Potential targets for drug development. *Drug Development Research* 49(3):187-200.

Donnelly-Roberts DL, Jarvis MF (2007). Discovery of P2X7 receptor-selective antagonists offers new insights into P2X7 receptor function and indicates a role in chronic pain states. *Br J Pharmacol* 151:571-579.

Duncan RL, Turner CH (1995). Mechanotransduction and the functional response of bone to mechanical strain. *Calcif Tissue Int.* 57 (5): 344-58.

Ericksen E, Moselkide L, Melsen F (1986). Trabecular bone remodeling and balance in primary hyperparathyroidism. *Bone* 7:213-221.

Fang J, Li Y, Zhang K, Zhao Z, Mei L (2016). Escaping the adverse impacts of NSAIDs on tooth movement during orthodontics. *Medicine* 95(16):e3256.

Feldman D (2013). Osteoporosis. 4th ed. Chapter 6 Osteoblast biology (pg. 93-130).: Elsevier Science.

Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B *et al.* (2006). Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nature genetics* 38(11):1310-1315.

Ferrari D, Pizzirani C, Adinolfi E, Lemoli M, Curti A, Idzko M (2006). The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol*;176:3877-3883.

Flanagan AM, Chambers TJ (1992). Stimulation of bone nodule formation *in vitro* by prostaglandins E<sub>1</sub> and E<sub>2</sub>. *Endocrinology* 130:443-448.

Funk CD (2001). Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* 294:1871-1875.

Gartland A, Ginty AF, Gallagher JA, Bowler WB (1999) Activation of P2X7 receptors expressed by human osteoclastoma modulates bone resorption. *Calci Tissue Int* 64:S56, abstract.

Gartland A, Buckley KA, Bowler WB, Gallagher JA (2003). Blockade of the pore-forming P2X7 receptor inhibits formation of multinucleated human osteoclasts *in vitro*. *Calcif Tissue Int.* 73:361-369.

Gartland A, Skarratt KK, Hocking LJ, Parsons C, Stokes L, Jorgensen NR (2012). Polymorphisms in the P2X7 receptor gene are associated with low lumbar spine bone mineral density and accelerated bone loss in post-menopausal women. *European journal of human genetics : EJHG* 20(5):559-564.

Genetos DC, Geist DJ, Liu D, Donahue HJ, Duncan RL (2005). Fluid shear-induced APT secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. *J Bone Miner Res.* 20:41-49.

Genetos DC, Karin NJ, Geist DJ, Donahue HJ, Duncan RL (2011). Purinergic signaling is required for fluid shear-stress induced NF- $\kappa$ B translocation in osteoblasts. *Exper Cell Research* 317(6):737-744.

Gennero I, Laurencin-Dalicieux S, Conte-Auriol F, Briand-Mesange F, Laurencin D, Rue J, Beton N, Malet N, Mus M, Tokumura A, Bourin P, Vico L, Brunel G, Oreffo R, Chun J, Salles JP (2011). Absence of the lysophosphatidic acid receptor LPA1 results in abnormal bone development and decreased bone mass. *Bone* 49:395-403.

George A, Sabsay B, Simonian PA, Veis A (1993). Characterization of a novel dentin matrix acidic phosphoprotein. Implications for induction of biomineralization. *The Journal of biological chemistry* 268(17):12624-12630.

Grol MW, Panupinthu N, Korcok J, Sims S, Dixon J (2009). Expression, signaling and function of P2X7 receptors in bone. *Purinergic Signaling* 5:205-221.

Grol MW, Zelner I, Dixon SJ (2012). P2X7-mediated calcium influx triggers a sustained PI3K-dependent increase in metabolic acid production by osteoblast-like cells. *Am J Physiol Endocrinol Metab* 302:E561-E575.

Grol MW, Pereverzev A, Sims SM, Dixon SJ (2013). P2 receptor networks regulate signaling duration over a wide dynamic range of ATP concentrations. *Journal of cell science* 126(Pt 16):3615-3626.

Guggino SE, Lajeunesse D, Wagner JA, Snyder SH (1989). Bone remodeling signaled by a dihydropyridine- and phenylalkylamine-sensitive calcium channel. *Proc Natl Acad Sci USA*. 86:2957-2960.

Harada S, Rodan GA (2003). Control of osteoblast function and regulation of bone mass. *Nature* 423(6937):349-355.

Hartsfield JK (2009). Pathways in external apical root resorption associated with orthodontia. *Orthod Craniofac Res* 12(3):236-242.

Heasley BH, Jarosz R, Lynch KR, Macdonald TL (2004). Initial structure-activity relationships of lysophosphatidic acid receptor antagonists: discovery of a high-affinity LPA1/LPA3 receptor antagonist. *Bioorg Med Chem Lett* 14:2735-2301.

Hipskind RA, Bilbe G (1998). MAP kinase signaling cascades and gene expression in osteoblasts. *Frontiers in bioscience : a journal and virtual library* 3:d804-816.

Huang W, Yang S, Shao J, Li YP (2007). Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Front Biosci* 12:3068-3092.

Issack PS, Helfet DL, Lane JM (2008). Role of Wnt signaling in bone remodeling and repair. *HSS journal : the musculoskeletal journal of Hospital for Special Surgery* 4(1):66-70.

Jacobson KA, Jarvis MF, Williams M (2002). Purine and pyrimidine (P2) receptors as drug targets. *J Med Chem*; 45(19):4057-4093.

Jiang L, Baldwin JM, Roger S, Baldwin SA (2013). Insights into the molecular mechanisms underlying mammalian P2X7 receptor functions and contributions in diseases, revealed by structural modeling and single nucleotide polymorphisms. *Front Pharmacol*. 55(4):1-17.

Jones DB, Bingmann D (1991). How do osteoblasts respond to mechanical stimulation? *Cells Mater* 1:329-340.

Jones DB, Nolte H, Scholubbers JG, Turner E, Veltel D (1991). Biochemical signal transduction of mechanical strain in osteoblast-like cells. *Biomaterials* 12:101-110.

Jorgensen NR, Henriksen Z, Sorensen OH, Eriksen EF, Civitelli R, Steinberg TH (2002). Intercellular calcium signaling occurs between human osteoblasts and osteoclasts and requires activation of osteoclast P2X7 receptors. *J Biol Chem* 277:7574-7580.

Kale S, Kocadereli I, Atilla P, Asan E (2004). Comparison of the effects of 1,25 dihydroxycholecalciferol and prostaglandin E<sub>2</sub> on orthodontic tooth movement. *Am J Orthod Dentofacial Orthop* 125:607-614.

Ke HZ, Qi H, Weidema AF, Zhang Q, Panupinthu N, Crawford DT, Grasser WA, Paralkar VM, Li M, Audoly LP, Gabel CA, Jee WS, Dixon SJ, Sims SM, Thompson DD (2003). Deletion of the P2X7 nucleotide receptor reveals its regulatory roles in bone formation and resorption. *Mol Endocrinol* 17:1356-67.

Klein DC, Raisz LG (1970). Prostaglandins: stimulation of bone resorption in tissue culture. *Endocrinology* 86:1436-1440.

Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrughe B, Nakashima K *et al.* (2005). NFAT and Osterix cooperatively regulate bone formation. *Nat Med* 11(8):880-885.

Krishnan V, Davidovitch Z (2006). Cellular, molecular, and tissue-level reactions to orthodontic force. *American journal of orthodontics and dentofacial orthopedics* 129(4):469 e461-432.

Krishnan V, Davidovitch Z (2009). On a path to unfolding the biological mechanisms of orthodontic tooth movement. *J of Dent Research* 88(7):597-608.

Kubota T, Michigami T, Ozono K (2009). Wnt signaling in bone metabolism. *Journal of bone and mineral metabolism* 27(3):265-271.

Labasi JM, Petrushova N, Donovan C, McCurdy S, Lira P, Payette MM, Brissette W, Wicks JR, Audoly L, Gabel CA (2002). Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol*; 168(12):6436-6445.

Lapierre DM, Tanabe N, Pereverzev A, Spencer M, Shugg RP, Dixon SJ, Sims SM (2010). Lysophosphatidic acid signals through multiple receptors in osteoclasts to elevate cytosolic calcium concentration, evoke retraction and promote cell survival. *J Biol Chem* 285:25792-25801.

- Lazarowski ER, Sesma JI, Seminario-Vidal L, Kreda SM (2011). Molecular Mechanisms of Purine and Pyrimidine Nucleotide Release. *Advances in Pharmacology* 61:221-247.
- Lazarowski ER (2012). Vesicular and conductive mechanisms of nucleotide release. *Purinergic Signaling*; 8(3):359-373.
- Lee W (1990). Experimental study of the effect of prostaglandin administration on tooth movement with particular emphasis on the relationship to the method of PGE1 administration. *Am J Orthod Dentofacial Orthop* 98:238-241.
- Leiker BJ, Nanda RS, Currier GF, Howes RI, Sinha PK (1995). The effects of exogenous prostaglandins on orthodontic tooth movement in rats. *Am J Orthod Dentofacial Orthop* 108:380-388.
- Li J, Liu D, Ke HZ, Duncan RL, Turner CH (2005). The P2X7 nucleotide receptor mediates skeletal mechanotransduction. *J Biol Chem* 280:42952-42959.
- Lister MF, Sharkey J, Sawatzky DA, Hodgkiss JP, Davidson DJ, Rossi AG (2007). The role of the purinergic P2X7 receptor in inflammation. *J Inflamm* 4:5.
- Liu D, Genetos DC, Shao Y, Geist DJ, Li J, Ke HZ, Turner CH, Duncan RL (2008). Activation of extracellular-signal regulated kinase (ERK1/2) by fluid shear is Ca<sup>2+</sup>-and ATP-dependent in MC3T3-E1 osteoblasts. *Bone* 42:644-652.
- Liu YB, Kharode Y, Bodine P, Yaworsky P, Robinson J, Billiard J (2010). LPA induces osteoblast differentiation through interplay of two receptors: LPA1 and LPA4. *J Cell Biochem* 109:794-800.
- MacDougall M, Gu TT, Luan X, Simmons D, Chen J (1998). Identification of a novel isoform of mouse dentin matrix protein 1: spatial expression in mineralized tissues. *Journal of bone and mineral research* 13(3):422-431.
- Masiello LM, Fotos JS, Galileo DS, Karin NJ (2006). Lysophosphatidic acid induces chemotaxis in MC3T3-E1 osteoblastic cells. *Bone* 39:72-82.
- Masella RS, Meister M (2006). Current concepts in the biology of orthodontic tooth movement. *Am J Orthod Dentofacial Orthop* 129:458-468.
- McCabe LR, Kockx M, Lian J, Stein J, Stein G (1995). Selective expression of fos- and jun-related genes during osteoblast proliferation and differentiation. *Exp Cell Res* 218(1):255-262.
- McMichael BK, Meyer SM, Lee BS (2010). C-Src-mediated phosphorylation of thyroid hormone receptor-interacting protein 6 (TRIP6) promotes osteoclast sealing zone formation. *J Biol Chem* 285:26641-26651.

- Meeran NA (2012). Biological response at the cellular level within the periodontal ligament on application of orthodontic force – An update. *J Ortho Sci* 1(1):2-10.
- Mehrotra M, Saegusa M, Voznesensky O, Pilbeam C (2006). Role of Cbfa1/Runx2 in the fluid shear stress induction of COX-2 in osteoblasts. *Biochem and Biophys Research Comm* 341(4):1225-1230.
- Minguell JJ, Erices A, Conget P (2001). Mesenchymal stem cells. *Exp Biol Med* 226:507-520.
- Morrison MS, Turin L, King BF, Burnstock G, Arnett TR (1998). ATP is a potent stimulator of the activation and formation of rodent osteoclasts. *J Physiol (Lond)*. 511:495-500.
- Na M (2016). Dexamethasone regulates gene expression induced by the mechanosensitive P2X7 receptor in osteoblasts (*unpublished Master's thesis*).
- Naemsch LN, Weidema AF, Sims SM, Underhill TM, Dixon SJ (1999). P2X4 purinoceptors mediate an ATP-activated, non-selective cation current in rabbit osteoclasts. *J Cell Sci* 112:4425-4435.
- Nelson DW, Gregg RJ, Kort ME, Perez-Medrano A, Voight EA, Wang Y, Grayson G, Namovic MT, Donnelly-Roberts DL, Niforatos W, Honore P, Jarvis MF, Faltynek CR, Carroll WA (2006). Structure-activity relationship studies on a series of novel, substituted 1-benzyl-5-phenyltetrazole P2X7 antagonists. *J Med Chem* 49:3659-3666.
- Nobuaki N, Kinoshita M, Yamaguchi K, Shima N, Yasuda H, Yano K, Morinaga T, Higashio K (1998). RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem and Biophys Research Communications* 253(2):395-400.
- Nochi H, Tomura H, Tobo M, Nobuyuki T, Sato K, Shinozaki T, Kobayashi T, Takagishi K, Ohta H, Okajima F, Tamoto K (2008). Stimulatory role of lysophosphatidic acid in cyclooxygenase-2 induction by synovial fluid of patients with rheumatoid arthritis in fibroblast-like synovial cells. *J Immunol* 181:5111-5119.
- North RA (2002). Molecular physiology of P2X receptors. *Physiol Rev*;82:1013-1067.
- Novack DV, Teitelbaum SL (2008). The osteoclast: friend or foe? *Annual review of pathology* 3:457-484.
- Ohlendorff SD, Tofteng CL, Jensen JE, Petersen S, Civitelli R, Fenger M, Abrahamsen B, Hermann A, Eiken P, Jorgensen NR (2007). Single nucleotide polymorphisms in the P2xP27 genes are associated to fracture risk and to effect of estrogen treatment. *Pharmacogenet Genomics* 17:555-567.



- Okumura H, Shiba D, Kubo T, Yokoyama T (2008). P2X7 receptor as sensitive flow sensor for ERK activation in osteoblasts. *Biochem Biophys Res Commun* 372:486-490.
- Orriss IR, Knight GE, Ranasinghe S, Burnstock G, Arnett TR (2006). Osteoblast responses to nucleotides increase during differentiation. *Bone* 39:300-309.
- Orriss IR, Knight GE, Utting JC, Taylor SEB, Burnstock G, Arnett TR (2009). Hypoxia stimulates vesicular ATP from rat osteoblasts. *J Cell Physiol.* 220:155-162.
- Orriss IR, Key ML, Brandao-Burch A, Patel JJ, Burnstock G, Arnett TR (2012). The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: The role of p2x receptors. *Bone* 51(3):389-400.
- Panupinthu N, Zhao L, Possmayer F, Ke HZ, Sims SM, Dixon SJ (2007). P2x7 nucleotide receptors mediate blebbing in osteoblasts through a pathway involving lysophosphatidic acid. *J Biol Chem* 282:3403-3412.
- Panipinthu N, Rogers JT, Zhao L, Solano-Flores LP, Possmayer F, Sims SM, Dixon SJ (2008). P2X7 receptors on osteoclasts couple to production of lysophosphatidic acid: a signaling axis promoting osteogenesis. *J Cell Biol* 181:859-871.
- Pereverzev A, Komarova SV, Korok J, Armstrong S, Tremblay GB, Dixon SJ, Sims SM (2008). Extracellular acidification enhances osteoclast survival through an NFAT-independent, protein kinase C-dependent pathway. *Bone* 42:150-161.
- Proffit WRF, H.W. Sarver, D.M. (2012). *Contemporary Orthodontics*. 5th ed. St. Louis: Mosby.
- Raisz LG, Sandberg AL, Goodson JM, Simmons HA, Mergenhagen SE (1974). Complement-dependent stimulation of prostaglandin synthesis and bone resorption. *Science*;185:789-791.
- Reitan KR. Principles and biomechanical reactions (1994). In: Graber TM, Vanarsdall RL, editors. *Orthodontics: current principles and techniques*. St. Louis: Mosby. p 88-174.
- Roberts WE (1989). Cell kinetics of the periodontal ligament. In: *The Biology of Tooth Movement*. NLB CJ editor. Boca Raton, FL: CRC Press, pp. 55-69.
- Roberts WE, Huja S, Roberts JA (2004). Bone modeling: biomechanics, molecular mechanisms and clinical perspectives. *Semin Orthod*;10:123-161.
- Roberts WE. Bone physiology, metabolism and biomechanics in orthodontic practice (2005). In: Graber TM, editor. *Orthodontics: current principles and techniques*. St Louis: Mosby; p 287-344.

Robling AG, Castillo AB, Turner CH (2006). Biomechanical and molecular regulation of bone remodeling. *Annual review of biomedical engineering*. 8:455-498.

Robling AG, Turner CH (2009). Mechanical signaling for bone modeling and remodeling. *Critical reviews in eukaryotic gene expression* 19(4):319-338.

Romanello M, Pani B, Bicego M, D'Andrea P (2001). Mechanically induced ATP release from human osteoblastic cells. *Biochem Biophys Commun*. 289:1275-1281.

Romanello M, Codognotto A, Bicego M, Pines A, Tell G, D'Andrea P (2005). Autocrine/paracrine stimulation of purinergic receptors in osteoblasts: contribution of vesicular ATP release. *Biochemical and biophysical research communications* 331(4):1429-1438.

Rouzer CA, Marnett LJ (2009). Cyclooxygenases: structural and functional insights. *J Lipid Res* 50:S29-S34.

Rubin J, Murphy TC, Zhu L, Roy E, Nanes M, Fan X (2003). Mechanical strain differentially regulates endothelial Nitric-Oxide Synthase and Receptor Activator of Nuclear K $\beta$  ligand expression via ERK1/2 MAPK. *J Biol Chem* 278:34018-34025.

Salles JP, Laurencin-Dalieux S, Conte-Auriol F, Briand-Mesange F, Gennero I (2013). Bone defects in LPA receptor genetically modified mice. *Biochimica et Biophysica Acta*. 1831:93-98.

Schwarz A (1932). Tissue changes incident to orthodontic tooth movement. *Int J Orthod* 18):331-352.

Sheng X, Ung Y, Chen A, Chun J (2015). Lysophosphatidic acid signaling in development. *Development* 142:1390-1395.

Sims MS, Panupinthu N, Lapierre D, Pereverzev A, Dixon SJ (2013). Lysophosphatidic acid: A potential mediator of osteoblast-osteoclast signaling in bone. *BBA-Molecular and Cell Bio of Lipids* 1881(1):109-116.

Sugimoto N, Takuwa N, Yoshioka K, Takuwa Y (2006). Rho-dependent, Rho-kinase independent inhibitory regulation of Rac and cell migration by LPA1 receptor in Gi-inactivated CHO cells. *Exper Cell Research* 312(10):1899-1908.

Suda M, Tanaka K, Yasoda A, Natsui K, Sakuma Y, Tanaka I, Ushikubi F, Narumiya S, Nakao K (1998). Prostaglandin (PGE<sub>2</sub>) Autoamplifies its Production Through EP1 Subtype of PGE Receptor in Mouse Osteoblastic MC3T3-E1 Cells. *Calcif Tissie Int*. 62:327-331.

Sun L, Blair HC, Peng Y, Zaidi N, Adebajo OA, Wu XB *et al.* (2005). Calcineurin regulates bone formation by the osteoblast. *Proceedings of the National Academy of Sciences of the United States of America*; 102(47):17130-17135.

Surprenant A, Rassendren F, Kawashima E, North RA, Buell G (1996). The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* 272(5262):735-738.

Suzuki A, Kotoyori J, Oiso Y, Kozawa O (1993). Prostaglandin E<sub>2</sub> is a Potential Mediator of Extracellular ATP Action in Osteoblast-like Cells. *Cell Adhesion and Communication* 1:113-119.

Suzuki A, Shinoda J, Oiso Y, Kozawa O (1995). Mechanism of phospholipase D activation induced by extracellular ATP in osteoblast-like cells. *J Endocrinol.* 145:81-86.

Taketa T, Sakai A, Tanaka S, Nakai K, Menuki K, Yamane H, Tanaka K, Nakamura T (2008). Selective cyclooxygenase-2 inhibitor prevents reduction of trabecular bone mass in collagen-induced arthritic mice in association with suppression of RANKL/OPG ratio and IL-6 mRNA expression in synovial tissues but not in bone marrow cells. *J Bone Miner Metab* 26:143-151.

Teitelbaum SL, Ross FP (2003). Genetic regulation of osteoclast development and function. *Nature reviews Genetics* 4(8):638-649.

Tian XY, Zhang Q, Zhao R, Setterberg RB, Zeng QQ, Iturria SJ, Ma YF, Jee WSS (2008). Continuous PGE<sub>2</sub> leads to net bone loss while intermittent PGE<sub>2</sub> leads to net bone gain in lumbar vertebral bodies of adult female rats. *Bone* 42:914-920.

Toyosawa S, Oya K, Sato S, Ishida K (2012). [Osteocyte and DMP1]. *Clinical calcium* 22(5):713-720.

Turner CH, Pavalko FM (1998). Mechanotransduction and functional response of the skeleton to physical stress: The mechanisms and mechanics of bone adaptation. *J Orthop Sci* 3:346-355.

Viecilli R, Katona T, Chen J, Roberts E, Hartsfield J, Jr. (2009a). Comparison of dentoalveolar morphology in WT and P2X7R KO mice for the development of biomechanical orthodontic models. *Anatomical record* 292(2):292-298.

Viecilli RF, Katona TR, Chen J, Hartsfield JK, Jr., Roberts WE (2009b). Orthodontic mechanotransduction and the role of the P2X7 receptor. *American journal of orthodontics and dentofacial orthopedics* 135(6):694 e691-616; discussion 694-695.

Volonte C, Amadio S, D'Ambrosi N, Colpi M, Burnstock G (2006). P2 receptor web: complexity and fine-tuning. *Pharmacology & therapeutics* 112(1):264-280.

Wadhwa S, Godwin SL, Peterson DR, Epstein MA, Raisz LG, Pilbeam CC (2002). Fluid Flow Induction of Cyclo-oxygenase 2 Gene Expression in Osteoblasts is Dependent on an Extracellular Signal-Regulated Kinase Signaling Pathway. *J Bone Miner Research* 17(2):266-274.

Watanabe-Tomita Y, Suzuki A, Shinoda J, Oiso Y, Kozawa O (1997). Arachidonic acid release induced by extracellular ATP in osteoblasts: role of phospholipase D. *Prostaglandins Leukot Essent Fatty Acids* 57:335-339.

Wang ZQ, Ovitt C, Grigoriadis AE, Mohle-Steinlein U, Ruther U, Wagner EF (1992). Bone and haematopoietic defects in mice lacking *c-fos*. *Nature* 360:741-745.

Waters KM, Tan R, Genetos DC, Verma S, Yellowley CE, Karin NJ (2007). DNA microarray analysis reveals a role for lysophosphatidic acid in the regulation of anti-inflammatory genes in MC3T3-E1 cells. *Bone* 41:833-841.

Wolff J (1892). The law of bone transformation Berlin: A Hirschwald.

Yamasaki K, Shibata Y, Imai S, Tani Y, Shibasaki Y, Fukuhara T (1984). Clinical application of prostaglandin E1 (PGE1) upon orthodontic tooth movement. *American journal of orthodontics* 85(6):508-518.

Yoshida KH, Oida T, Kobayashi T, Maruyama M, Tanaka T, Katayama K, Yamaguchi E, Segi T, Tsuboyama M, Matusushita (2002). Stimulation of bone formation and prevention of bone loss by prostaglandin E EP4 receptor activation. *Proc Natl Acad Sci, USA*. 99:4580-4585.

You J, Reilly GC, Zhen X, Yellowley CE, Chen Q, Donahue HJ, Jacobs CR (2001). Osteopontin Gene Regulation by Oscillatory Fluid Flow via Intracellular Calcium Mobilization and Activation of Mitogen-activated Protein Kinase in MC3T3-E1 Osteoblasts. *J Biol Chem* 276:13365-13371.

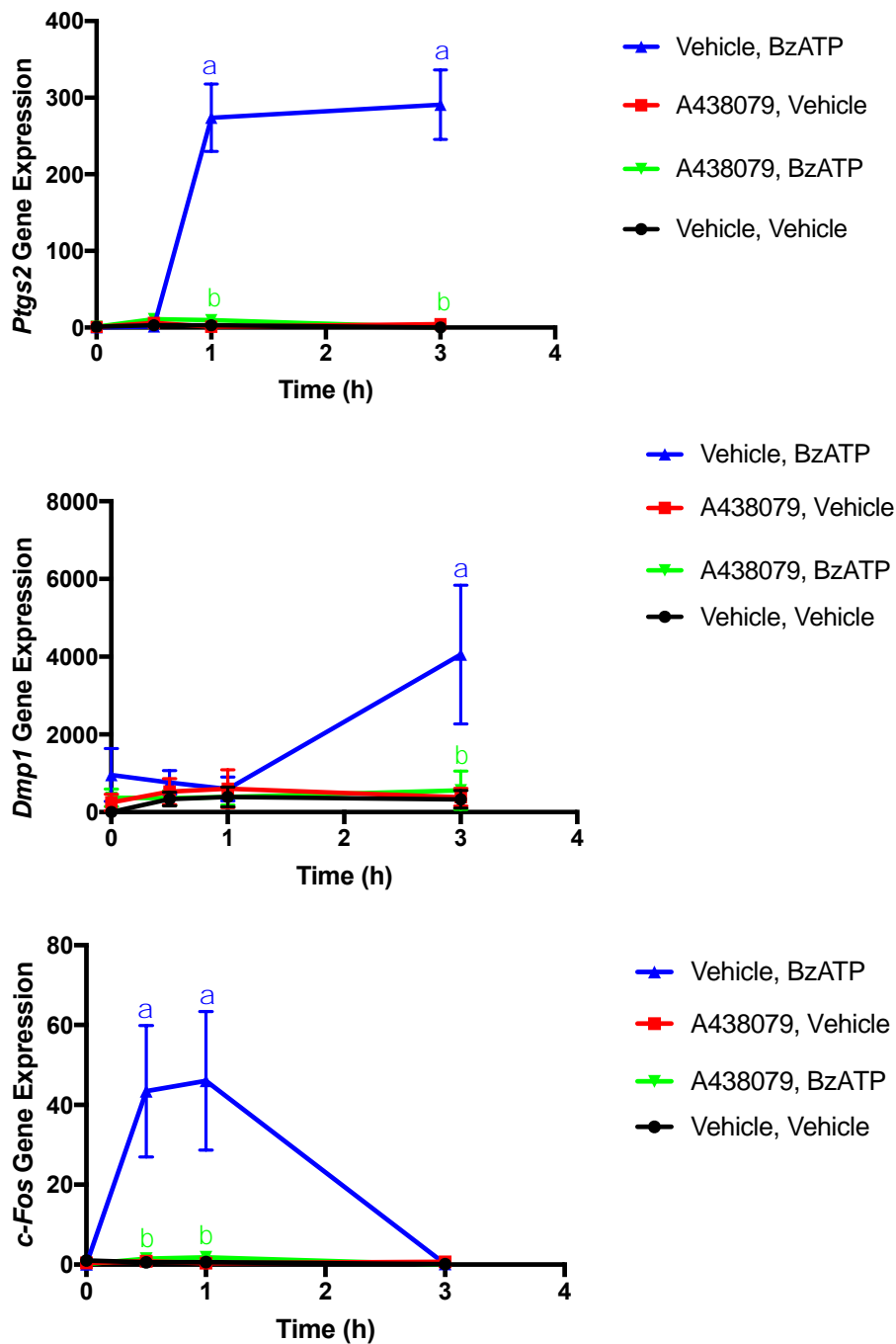
Yung Y, Stottard N, Chun J (2014). LPA receptor signaling: pharmacology, physiology and pathophysiology. *J Lipid Research* 55:1192-1214.

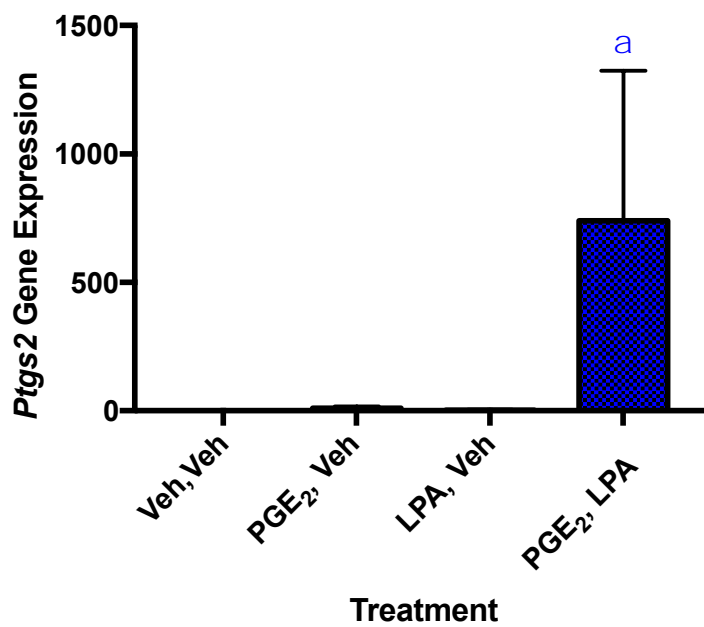
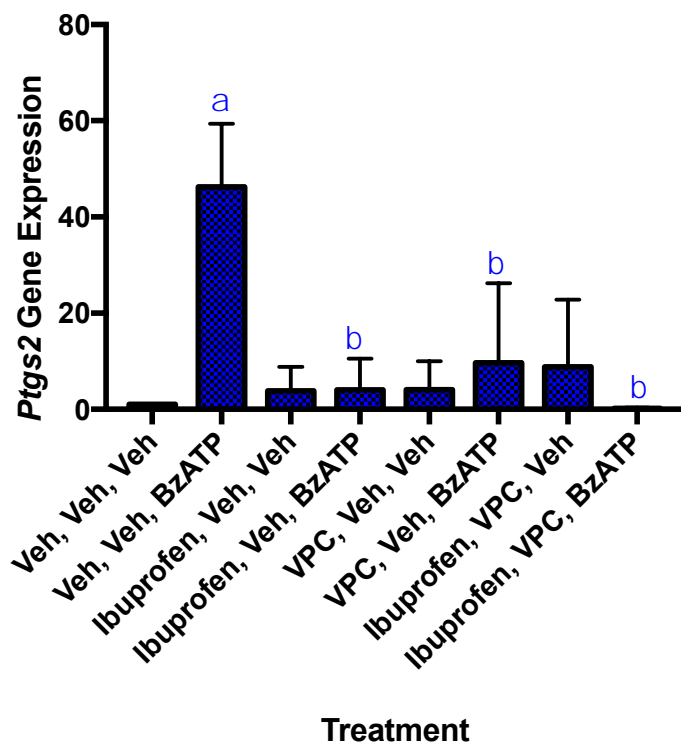
Zhang P, Wu Y, Jiang Z, Jiang L, Fang B (2012). Osteogenic response of mesenchymal stem cells to continuous mechanical strain is dependent on ERK 1/2-Runx 2 signaling. *Int J of Mol Med* 29(6):1083-1089.

## APPENDICES

**Appendix A: Results presented without the normalization as percentage of maximum gene expression.**

Data are shown as relative gene expression of respective genes





## Appendix B: Permission to use Figure 2.

License Number	3925021092457
License date	Aug 09, 2016
Licensed Content Publisher	Springer
Licensed Content Publication	Purinergic Signalling
Licensed Content Title	Expression, signaling, and function of P2X7 receptors in bone
Licensed Content Author	Matthew W. Grol
Licensed Content Date	Jan 1, 2009
Licensed Content Volume	5
Licensed Content Issue	2
Type of Use	Thesis/Dissertation
Portion	Figures/tables/illustrations
Number of figures/tables/illustrations	1
Author of this Springer article	No
Order reference number	
Original figure numbers	Figure 10. Proposed role for P2X7 receptors in mechanotransduction in bone.
Title of your thesis / dissertation	Role of lipid mediators in P2X7 signaling in osteoblasts
Expected completion date	Jan 2017
Estimated size(pages)	50

## CIRRICULUM VITAE

**Name:** Erin Eyer

**Post-secondary Education and Degrees:**

University of Manitoba  
Winnipeg, Manitoba, Canada  
2000-2003 B.Sc.

University of Manitoba  
Winnipeg, Manitoba, Canada  
2003-2007 D.M.D.

Western University  
London, Ontario, Canada  
2014-2017 M.C.I.D.

**Honours and Awards:**

Dr. Frances Matiowsky Memorial Award  
University of Manitoba  
2007

Manitoba Dental Association Scholarship  
University of Manitoba  
2006

Leonard Krueger Scholarship  
University of Manitoba  
2004

**Related Work Experience:**

Private Practice  
Stonewall, Manitoba, Canada  
2008-2014

Private Practice  
Winnipeg, Manitoba, Canada  
2007-2008