Continuous PTH: A Tale of Two Pathways

Thomas Estus
University of Connecticut - Storrs, thomas.estus@uconn.edu

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Abstract

Parathyroid hormone (PTH) is a key factor in bone metabolism, able to stimulate both osteoblasts to cause bone formation and osteoclasts to cause bone resorption. The studies presented herein set forth to examine both the formative and resorptive pathways in light of a newly discovered inhibitory factor, serum amyloid a3 (Saa3). Saa3 is particularly germane to the PTH signaling pathway as it is secreted by cells of the osteoclast lineage in response to PTH stimulation of prostaglandin. Saa3 then enacts its inhibitory effects by acting back onto osteoblasts to inhibit PTH’s ability to stimulate cAMP/PKA signaling. In the absence of Saa3 we have observed that PTH is able to stimulate the phosphorylation of β-catenin at two novel sites, serine 552 and serine 675. These sites represent a cAMP/PKA dependent mechanism by which β-catenin signaling may be stimulated outside the dogma of canonical wnt signaling. Our data suggest that PTH phosphorylation of these sites increases β-catenin signaling as measured by TCF/LEF activity and downstream gene expression, and that the inclusion of Saa3 into these conditions completely ablates this pathway. PTH is also known to stimulate receptor activator of nuclear kappa-b ligand (RANKL) expression by osteoblasts via cAMP/PKA. As a potent stimulator of osteoclastogenesis we were then interested to examine the effects of the cAMP inhibitor, Saa3, upon PTH stimulation of RANKL. Interestingly, we discovered that PTH stimulation of RANKL was not only unaffected by inhibition of the cAMP/PKA pathway, but that antagonists of the Ca^{2+}/PKC
pathway were able to prevent PTH stimulation of RANKL even in the presence of a robust cAMP response to PTH. We then conclude that the anabolic portions of the PTH pathway are mediated through cAMP/PKA and the catabolic portions are mediated through Ca\(^{2+}\)/PKC. This dichotomy explains how the catabolic effects of PTH are more visible and pronounced as they go unhindered by the inherent Saa3 negative feedback loop which renders the anabolic effects invisible in basal conditions.
Continuous PTH: A Tale of Two Pathways

Thomas Lacey Estus

B.S., California Lutheran University

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
at the
University of Connecticut

2015
APPROVAL PAGE

Doctor of Philosophy Dissertation

Continuous PTH: A Tale of Two Pathways

Presented by
Thomas Lacey Estus, B.S.

Major Advisor ________________________________________________________________
Carol Pilbeam

Associate Advisor ___________________________________________________________
Douglas Adams

Associate Advisor ___________________________________________________________
Anne Delaney

Associate Advisor ___________________________________________________________
Yusuf Khan

Associate Advisor ___________________________________________________________
Mina Mina

University of Connecticut
2015
Acknowledgments

First I would like to thank Carol Pilbeam and Shilpa Choudhary for their amazing mentorship and support. They were always patient with me and showed true compassion during the time I spent in the lab. I could always count on both of you to be there to discuss research or simply share a pleasant conversation. I will miss you both tremendously. I would also like to thank my other committee members: Doug Adams, Anne Delaney, Mina Mina, and Yusuf Khan. Their guidance during my tenure was invaluable.

My labmates and dear friends: Lyndon, Cheryl, and Jessica; I cannot thank you enough for all of your support as I trudged through. Not only were you an inspiration to me as scientists, but I was lucky enough to call you friends. I could always count on you guys to bounce ideas off of, either for new experiments or how to deal with personal matters. I truly miss our lunch outings and general debauchery. Never forget the Wahib.

My friend and brother Vinnie. Thank you for providing a musical balance, and letting me escape the lab to come perform with you during the weekends. It meant the world to me that I was able to continue to indulge in my passion for performance. Without your generosity in including me I would have had to hang it up long ago. Our many adventures both remembered and misremembered in and around New England. They were not ready for that LA flavor. Thanks also to you and your family for adopting me during the holidays.

My home gaming group: Ryan, Scott, John, and Spencer. Thanks for coming along on all the crazy adventures I put you through over hangouts. I could not do it without you, and the creative outlet you allow me to explore was a true foundation to hold onto myself. I look forward to seeing how it all ends! Ryan thank you also for your neverending postcards. Each and every one brought a smile to my face and reminded me of what was important.

My local gaming group: Corwin, Jesse, Anna, Nick, Andrew, Bryan, Amy, Steve. Thank you for being there on the regular to share all the ups and downs of our adventures. I could not have met a better group to delve dungeons with.

J.B. thank you for teaching me how to snowboard and your unending hospitality. Your warmth as a person and love of life knows no equal.

Mitchell, baby you know I love your way.

All of the professors within the SCOB program. Thank you for adopting me into your program and sharing all of your knowledge and experience; without which this work would not have been possible.

Finally, thank you to my parents for raising me to be the person I am today. I do my best to take the love you invested in me and share it with the world.
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1. Introduction

1.1 Bone Homeostasis: Osteoblasts and Osteoclasts

Bone homeostasis is regulated by two cell types: osteoblasts, which form bone, and osteoclasts, which resorb bone. During development, bone grows by the process of modeling, in which resorption and formation can occur independently. In adults, the bone remodeling cycle—bone resorption followed by bone formation—determines the maintenance of bone mass. This cycle is known as bone turnover. An imbalance or uncoupling of this cycle, resulting in resorption being greater than formation, causes bone loss. Hence, much of our attempts to treat or prevent osteoporosis center on trying to manipulate the bone remodeling cycle.

Osteoblasts are differentiated from mesenchymal precursor cells. Once cells are committed to the osteoblastic lineage, they can differentiate into mature osteoblasts, which secrete matrix that can be subsequently mineralized to form bone. Active osteoblasts subsequently become quiescent cells lining the mineralized matrix, but they may be reactivated to form new matrix by certain agents or to uncover the mineralized matrix to allow resorption. The terminal cell of the osteoblast lineage is the osteocyte. Osteocytes are non-replicating cells that reside within the mineralized matrix. They are connected to each other and to cells on the surface by an extensive network of dendritic outgrowths through small channels called canaliculi. The osteocyte is thought to be the cell that senses and communicates mechanical strain. Recent studies suggest that the osteocyte is also important in mediating the regulation of the bone turnover cycle.

Osteoclasts are multinucleated cells of the hematopoietic lineage able to resorb mineralized tissue. Formation and differentiation of osteoclasts from hematopoietic
precursor cells requires two factors: 1) macrophage colony-stimulating factor (M-CSF) and 2) receptor activator of nuclear kappa-b ligand (RANKL). When exposed to these two factors osteoclast precursors proliferate and fuse into mature osteoclasts. Mature osteoclasts can then initiate resorption by first eroding the inorganic components of the bone in a process known as demineralization. This phase is followed by degradation of the organic components, primarily the type I collagen matrix. Osteoclasts achieve this resorption by creating an isolated acidic environment between their cellular membrane and where it attaches to the bone surface. Hallmarks of this process include a ruffled membrane morphology as well as pits of resorption left behind on the bone surface.

Osteoblasts and osteoclasts are not merely linked by their opposing actions on bone metabolism, but have a direct connection via RANKL. RANKL expressed by osteoblasts is able to stimulate osteoclasts through its receptor RANK, and RANK signaling is directly responsible for osteoclastogenesis. RANKL is expressed by several cell types, but seems to require cell-cell contact despite there being a soluble form, relegating RANKL to local action only \(^{(3, 4)}\). In fact, RANKL membrane shedding has been shown to decrease osteoclastogenesis \(^{(5)}\). Osteoblasts can also secrete osteoprogeterin, a decoy receptor for RANKL, in order to antagonize RANKL stimulated osteoclastogenesis. Parathyroid hormone (PTH) is able to stimulate both resorption and formation by stimulating the differentiation of osteoblasts and their subsequent RANKL expression – causing the differentiation of osteoclasts. Diseases such as osteoporosis and osteitis fibrosa are direct symptoms of an imbalance in formation and resorption rates of these cells, in some cases these diseases result from pathological conditions affecting physiological levels of PTH \(^{(6-8)}\).
1.2 Parathyroid Hormone

PTH is an eighty-four amino acid long peptide produced in the parathyroid by chief cells (9,10). PTH maintains physiological levels of calcium within the blood by regulating bone resorption and formation, increasing 1,25(OH)_{2}D_{3} production in the kidney, and by regulating Ca^{++} reabsorption. PTH acts via a G-protein coupled receptor called PTH1R that is highly expressed by osteoblasts and in the kidney. The intracellular signaling pathways activated via the PTH1R receptor include G_{s}-mediated activation of adenylate cyclase, resulting in cAMP production and protein kinase A (PKA) activation, and G_{q/11}-mediated phospholipase-C-β (PLCβ) stimulation, leading to inositol 1,4,5-trisphosphate (IP_{3}) production, calcium mobilization, and protein kinase C (PKC) activation (11,12). PTH can act anabolically on bone by modulating osteoblast proliferation, differentiation, and survival, (13,14); and catabolically, by promoting osteoclast formation via osteoblastic RANKL expression (15,16). The anabolic and catabolic effects can be induced by intermittent or continuous treatment, respectively (17).

Studies have indicated that the anabolic effects of PTH occur largely through the cAMP signaling pathway (12,18). In order to stimulate cAMP production, PTH binds to its receptor, and in turn releases G_{s}. G_{s} then travels to the transmembrane protein adenylate cyclase (AC). Once bound, AC is able to catalyze the transition of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Binding of cAMP to the regulatory subunits of the tetramer PKA allows the catalytic subunits to phosphorylate downstream proteins such as cAMP response element-binding protein (CREB) in order to affect transcription directly or indirectly via nuclear translocation.
Alternatively, cAMP can then activate the guanine exchange factor exchange protein directly activated by cAMP (EPAC). EPAC, in turn, converts the GDP bound to the GTPase Ras-related protein (Rap) to GTP. Rap is then able to bind to the kinase B-Raf, which in turn signals through the mitogen activated kinase MEK/extracellular signal-regulated kinase (ERK) pathway to affect a host of cellular responses including: survival, proliferation, and differentiation (19-21).

Two modes of PTH receptor activation exist: 1) a short-term activation of the receptor and 2) sustained activity of the receptor caused by receptor internalization (22). Sustained activity occurs contrary to canonical GPCR signaling—in which internalization of the GPCR prevents additional receptor activation from occurring. In sustained activity the PTH receptor continues to stimulate sustained levels of cAMP production once internalized in an endosomal compartment containing PTH, G-protein subunits, and adenylate cyclase (23, 24).

1.3 Cyclooxygenase-2

PTH can also induce expression of COX-2 in osteoblasts (25-27). COX-2 is an isozyme of the cyclooxygenase family which contains two members: COX-1 and COX-2. COX-1 is constitutively expressed, while COX-2 expression is inducible (28). Rate limiting COX enzymes catalyze the cyclooxygenase reaction that converts arachidonic acid into prostaglandin G₂ (PGG₂) via a cyclooxygenase reaction, and further into prostaglandin H₂ (PGH₂) via a peroxidase reaction (29). PGH₂ is the major precursor of several bioactive proteins, most notably in osteoblasts: prostaglandin E₂ (PGE₂) (25, 30).
In vivo studies in our lab show that basal concentrations of cAMP within the plasma of COX-2 KO mice are higher than in WT mice, implicating COX-2 as a factor that causes inhibition of cAMP production \(^{(31)}\).

1.4 Wnt/β-catenin Signaling

The members of the Wnt family of glycoproteins are typically 350-400 amino acids long. Their secretion accompanies effects upon many developmental processes including cell fate determination, cell polarity, limb patterning, proliferation, and migration \(^{(32, 33)}\). In addition, they are highly conserved across species and primarily affect cells through β-catenin dependent and independent pathways. The β-catenin dependent pathway is commonly referred to as the canonical pathway and involves a Wnt protein binding to a dimer comprised of isomers of the proteins frizzled (Fz) and low-density lipoprotein receptor-related protein (LRP). Once this binding occurs, the protein disheveled (Dsh) is activated. At resting state, β-catenin is quickly degraded via ubiquitinylation mediated by a destruction complex comprised of several proteins: Glycogen synthase kinase 3 beta (GSK3-β), Axin, and Adenomatous polyposis coli (APC). Once activated by Wnt-Fz-LRP interaction, Dsh inhibits GSK3-β from marking β-catenin for destruction. Following inhibition of the destruction complex, β-catenin accumulates in the nucleus where it acts to regulate TCF/LEF family transcription factors. These factors can target a wide variety of genes that in turn affect cell fate, survival, and proliferation \(^{(34)}\).

β-catenin is marked for destruction and stabilization by a hyper- and hypophosphorylated state, respectively. The destruction complex phosphorylates β-
β-catenin first on Ser45 via casein kinase 1 (CK1) action; resulting in phosphorylation of Thr41, Ser33, and Ser37. This hyperphosphorylation marks β-catenin for ubiquitylation by βTrcp to be proteasomally degraded. Conversely, when β-catenin is in its hypophosphorylated state, characterized by phosphorylation on either Ser552 or Ser675 or both, it is marked for stabilization and translocation to the nucleus. This is mediated by protein kinases including PKA and Akt, the former specific to Ser675 (35-38).

Wnt signaling that occurs independent of β-catenin is designated non-canonical signaling and can occur via several pathways including the Wnt/calcium pathway, the heterotrimeric GTP-binding protein pathway, and the planar cell polarity pathway (PCP) (34).

1.5 PTH modulates the Wnt signaling pathway via cAMP/PKA activation

Synthetic segments of PTH able to affect the cAMP/PKA pathway also increased expression of several Wnt signaling components including β-catenin, LRP-6, and Fz-1 when given continuously. In addition, decreases in LRP-5 and Dkk1, an LRP-6 inhibitory ligand, were also seen (39, 40). Both intermittent PTH treatment, as well as cAMP-dependent PKA activity were shown to inactivate GSK3-β via phosphorylation (18, 41). This suggests that the PKA activity upon GSK-3-β can be mediated by PTH, which is coupled directly to PKA via the PTH receptor. PTH receptor was also found to directly bind Dsh in order to stimulate β-catenin signaling independent of Wnts and LRPs (16). Constitutively active GSK-3-β abolishes TCF-dependent transactivation by forskolin. This affect was shown to be receptor independent as Dkk1 overexpression to out-compete LRP activation could not abolish forskolin mediated transcriptional regulation.
of RANKL \(^{(18)}\). Additionally, Dkk1 was found to inhibit PTH stimulated cAMP production \(^{(42)}\). It seems then that the canonical Wnt signaling pathway is inhibitory for RANKL expression as further studies focusing on models treated with Wnt3a or overexpressing β-catenin showed a decrease in PTH stimulated RANKL expression and the opposite was seen in models featuring Dkk1 treatment or the ablation of β-catenin expression \(^{(43-46)}\).

A novel PKA-dependent pathway in which PTH recapitulates the actions of Wnt ligands on LRP6 by forming a ternary complex with both its receptor and LRP6 has been observed \(^{(47)}\). LRP6’s intracellular domain is comprised of five PPSP motifs accompanied by CK1 sites. These motifs can be phosphorylated by PKA, GSK3-β, and CK1 \(^{(48,49)}\). LRP6 becomes able to recruit Axin away from the destruction complex once phosphorylated; thereby, preventing it from marking β-catenin for degradation. While typically LRP6 activation is seen in regard to the Wnt signaling pathway, it has also been shown that PTH is able to not only cause phosphorylation of LRP6 at the PPSP motif corresponding to Ser1490, but form a ternary complex including PTH, PTH1R, and LRP6 \(^{(47)}\). This effect was shown to be dependent upon PKA, and specific to LRP6, as the same phenomena was not seen with LRP5 stimulated with PTH and forskolin, an adenylate cyclase agonist. This fits well with previous findings that LRP5 deficiency in mice did not inhibit the anabolic effects of PTH upon bone \(^{(50,51)}\). Additionally, continuous PTH activity has been shown to down-regulate mRNA expression of LRP5 while up-regulating that of LRP6 both in vitro and in vivo \(^{(39)}\).

1.6 PTH and RANKL
An essential component of the pathway by which PTH affects bone homeostasis is RANKL. Expressed by osteoblasts to stimulate osteoclastogenesis, RANKL has been traditionally thought to be stimulated by PTH via cAMP/PKA activation (52, 53). This notion is reinforced by the existence of several regulatory cAMP response elements upstream of the RANKL gene that control its expression (54, 55). Further evidence of the importance of the PTH/cAMP/PKA/RANKL interaction to bone homeostasis was seen when this region was deleted in mice, resulting in increased bone mass and a reduction in RANKL expression response to PTH treatment (56). However, alternative evidence points to a more complex interaction underlying PTH stimulation of RANKL.

PKA activation within mesenchymal stem cells was shown to decrease the RANKL/OPG ratio (57). Further, in mice lacking the receptor for oncostatin M cAMP response to PTH was unchanged while RANKL response was sustained, causing a catabolic resorption response to a normally anabolic PTH treatment modality; certainly suggesting that cAMP is not the only regulator of PTH/RANKL interaction (8). While previously shown that exhaustion of the PKC response system via pretreatment of PKC-agonist PMA has no effect on PTH’s ability to stimulate RANKL expression, an alternative study has shown that both PMA and ionomycin are capable of increasing RANKL expression pointing to involvement of both PKC and calcium in the RANKL pathway (53, 56). Both the involvement of PKA and PKC in the regulation of PTH stimulated RANKL expression was also shown when inhibitors of each pathway blunted the RANKL response to PTH (59). Moreover, it has been hypothesized that downstream
of PTH activation of the calcium/PKC pathway is activation of MEK/ERK, since inhibitors of both calcium and PKC signaling prevented ERK activation in osteoblasts\(^ {60}\).

Additionally, PTH effects upon RANKL expression and osteoclastogenesis are dependent upon prostaglandins. PTH stimulation of RANKL gene expression by osteoblasts was decreased in COX-2 KO osteoblast/osteoclast co-cultures resulting in decreased osteoclast formation\(^ {61}\). Similar results were seen in co-cultures lacking EP2, the receptor for PGE2\(^ {62}\). However, once again, prostaglandins do not represent the whole story as NSAID treatment blocked lipopolysaccharide induced PGE2 inhibition of OPG expression, but not that of RANKL\(^ {63}\). Alternate studies have also shown that PTH is able to increase RANKL expression and decrease that of OPG despite the presence or absence of either EP2 or EP4\(^ {64}\). Independent of PGE2, PTH analogs unable to stimulate cAMP production do not decrease OPG expression, but maintain their ability to stimulate RANKL\(^ {65}\).

*In vivo* evidence of the calcium and PKC pathway was shown when a low calcium diet given to mice with a mutation preventing PTHR activation of PLC, an important upstream event in the PKC cascade. These mice showed attenuated bone formation in response to PTH when compared to WT mice\(^ {66}\). Further, the PKC pathway seems to be important downstream of RANKL. The PKC inhibitor GF109203 blocks osteoclastogenesis and osteoclast activity and osteoclastic gene expression in RANKL treated bone marrow cultures\(^ {67}\). It has been hypothesized that the pathway downstream of RANKL focuses on PKC-β’s ability to inactivate GSK-3β allowing NFATc1 to induce osteoclastic gene expression\(^ {68}\). This hypothesis was supported by RANKL induced bone resorption in calvaria being decreased by PKC-β inhibition\(^ {68}\).
1.7 PTH and Serum Amyloid A3

Observations regarding the interaction between PTH and PGE2 revealed that in the absence of COX-2, the enzyme responsible for PGE2 production, the typical catabolic response expected during continuous PTH treatment was absent. In fact it was observed that in vivo continuous PTH infusion became anabolic and the anabolic effect of intermittent PTH treatment was increased in COX2 null mice\(^{(69)}\). Results from these in vivo are summarized in Figure 1. Evidence for the anabolic effects of PTH in Cox2 KO mice can be seen in elevated levels of P1NP in the serum (Fig. 1a), increases in osteoblast surface (Fig. 1g), ultimately culminating in an increase in femoral bone marrow density and volume (Fig. 1c-d). However, measures of bone resorption were similarly increased in both Cox2 KO and WT mice treated with PTH: serum CTX (Fig. 1b), osteoclast surface (Fig. 1h), and RANKL/OPG mRNA expression (Fig. 1e-f). Ultimately, this revealed a pathway by which Cox2 acts to antagonize the anabolic effects of PTH while leaving the catabolic response intact.

In vitro studies revealed that the PTH/PGE2 negative feedback loop required action by bone marrow macrophages (BMMs) stimulated down the osteoclast lineage by RANKL\(^{(70)}\). Ultimately this negative feedback loop was determined to be due to serum amyloid a3 (Saa3) secreted by osteoclast precursors in response to PTH stimulated PGE2 production by osteoblasts\(^{(71)}\). Once secreted, Saa3 acts back onto the N-formyl peptide receptor 2 (Fpr2) expressed by osteoblasts to inhibit the ability of PTH to stimulate cAMP production in a G\(\alpha_i/o\) dependent manner\(^{(71)}\).
Saa3 is an apolipoprotein which experiences acute and dramatic increases in its circulatory expression during inflammation (72-74). In mice, Saa3 is the only member of the serum amyloid a family to be expressed in extra-hepatic tissues; Saa1 and Saa2 expression is confined to the liver (74-77). Serum amyloids play roles in several diseases associated with inflammation, including: rheumatoid arthritis, atherosclerosis, obesity, cancer metastasis, and amyloidosis (77-79). In addition, Saa3 is expressed during the osteoblast-osteocyte differentiation stage and stimulates osteoclastic differentiation (80).

1.8 Specific Aims

Aim 1: Use COX-2 KO mice to investigate the actions of PTH on the Wnt signaling pathway in a system lacking Saa3. We hypothesize that conditioned medium containing Saa3 will inhibit PTH’s actions on the Wnt signaling pathway.

1A: Determine if PTH stimulated β-catenin phosphorylation and gene expression are mediated by the cAMP/PKA pathway

1B: Determine if conditioned medium containing Saa3 inhibits PTH-stimulated wnt target gene transcription.

Aim 2: Utilize Saa3 as a cAMP inhibitor to investigate the role of cAMP signaling in PTH stimulated RANKL expression. We hypothesize that PTH stimulated RANKL
expression acts via the PKC pathway and not the cAMP/PKA pathway in our model system.

2A: Determine the role of calcium and PKC in PTH stimulated RANKL expression in a system that cannot respond to PTH via cAMP.

2B: Determine the role of ERK signaling in the PTH/RANKL pathway.
Fig. 1. Skeletal response of Cox2 KO and WT mice after 12 days of PTH infusion (40 μg/kg/d). (A) serum marker of formation, P1NP, (B) serum marker of resorption, CTX, (C) Femoral BMD % change measured in vivo at beginning and end of infusion, (D) μCT morphometric analysis of femoral BV/TV, (E) tibial OPG mRNA expression, (F) tibial Rankl mRNA expression, (G) histomorphometric analysis of femoral Obs./BS, (H) histomorphometric analysis of femoral Ocs./BS. Bars are means ± SEM for 7 WT and 7 KO mice treated with vehicle and 8 WT and 8 KO mice treated with PTH. a Significant effect of PTH, p<0.01. b Significant effect of genotype, p<0.01.
2. Inhibition of PTH-stimulated β-catenin signaling in osteoblasts by bone marrow macrophages

2.1 Abstract

Bone marrow macrophages (BMMs), in the presence of cyclooxygenase-2 (Cox2) produce PGE₂, and secrete an inhibitory factor in response to Rankl that blocks PTH-stimulated osteoblastic differentiation. The goal of this study was to determine if the inhibitory factor also blocks PTH-stimulated Wnt signaling. Primary calvarial osteoblasts (POBs) were co-cultured with conditioned medium (CM) from Rankl-treated wild type (WT) BMMs, which make the inhibitory factor, and Cox2 knockout (KO) BMMs, which do not. PTH induced cAMP production was blocked by WT CM but not by KO CM. In the presence of KO CM, PTH induced phosphorylation at β-catenin serine sites, ser552 and ser675, previously shown to be phosphorylated by protein kinase A (PKA). Phosphorylation was blocked by WT CM and by H89, a PKA inhibitor. PTH did not increase total β-catenin. PTH-stimulated transcription factor/lymphoid enhancer-binding factor response element activity in POBs was blocked by WT CM and by serum amyloid A (SAA), the human recombinant analog of murine Saa3, which has recently been proposed to be the inhibitory factor. In POBs cultured with Cox2 KO CM, PTH increased expression of multiple genes associated with the anabolic actions of PTH and decreased expression of Wnt antagonists. This differential regulation of gene expression was not seen in POBs cultured with WT CM. These data highlight the ability of PTH to phosphorylate β-catenin directly via PKA and demonstrate the ability of a Cox2-dependent inhibitory factor, secreted by preosteoclasts, to abrogate PTH
stimulated β-catenin signaling. Our results suggest that PTH can stimulate a novel negative feedback of its anabolic actions by stimulating Rankl and Cox2 expression.

Key Words: Wnt genes, serine 552/675, cyclooxygenase-2, serum amyloid A, protein kinase A, cAMP
2.2 Introduction

Parathyroid hormone (PTH) is a potent regulator of bone homeostasis—able to stimulate both bone formation and resorption by stimulating the differentiation of both osteoblast and osteoclast populations \(^{(81,82)}\). Intermittent therapy with human 1-34 PTH (teriparatide) was the first FDA approved anabolic therapy for osteoporosis \(^{(83)}\). In contrast, continuous PTH administration or elevation has been shown to cause bone loss \(^{(82)}\). PTH acts via its receptor PTH1R, a G-protein coupled receptor that is highly expressed by osteoblasts and that activates both G\(_{\alpha_s}\) and G\(_{\alpha_i}\) signaling pathways \(^{(84)}\). G\(_{\alpha_s}\) activates adenylyl cyclase resulting in cAMP production and protein kinase A (PKA) activation, while G\(_{\alpha_i}\) leads to activation of protein kinase C (PKC) and release of Ca\(^{2+}\).

The anabolic effects of PTH are thought to be mediated via G\(_{\alpha_s}\) \(^{(12,85)}\). PTH is also known to increase the expression of cyclooxygenase 2 (Cox2), an inducible enzyme responsible for acute production of prostaglandin E\(_2\) (PGE\(_2\)), and PGE\(_2\) can also stimulate both bone formation and resorption \(^{(25,26,86,87)}\). In vitro studies have shown that PTH can stimulate osteoblast differentiation in Cox2 KO bone marrow stromal cells (BMSCs) but not in WT BMSCs \(^{(70)}\). In these studies, PGE\(_2\), produced either by Cox2 in osteoblasts or Cox2 in BMMs, acted via the EP4 receptor to cause bone marrow macrophages (BMMs), committed to the osteoclastic lineage by Rankl, to secrete a factor that inhibited the PTH stimulation of osteoblastic differentiation \(^{(70)}\). In vivo studies showed there was a greater anabolic effect of intermittent PTH in Cox2 KO mice compared to WT mice \(^{(31)}\). When PTH was administered continuously by infusion to WT mice, it was catabolic as expected. In contrast, PTH was markedly anabolic in
KO mice\(^{(69)}\). The inhibitory factor produced by the BMMs has recently been proposed to be serum amyloid A3 (Saa3)\(^{(71)}\).

cAMP-dependent PKA has been shown to cause the phosphorylation of β-catenin at two distinct sites: serine 552 (Ser552) and serine 675 (Ser675)\(^{(36,37)}\). These sites have been linked to increases in β-catenin mediated transcriptional activity. Traditionally, β-catenin has been linked with osteoblast differentiation via the canonical Wnt pathway, in which Wnt agonists act via Lrp5/6 and Frizzled receptors/co-receptors on the β-catenin-destruction-complex to prevent the ubiquitination and proteolysis of β-catenin\(^{(88)}\). The cAMP/PKA pathway provides for a novel Wnt-independent mechanism in which β-catenin proteins are acted upon directly to increase their signaling efficacy. PTH signaling has recently been shown to phosphorylate β-catenin at Ser552 in a cAMP-dependent manner and to increase the downstream transcriptional activity of β-catenin via this pathway\(^{(89)}\).

The goal of the current study was to investigate the effects of the Cox2-dependent inhibitor on PTH-stimulated β-catenin signaling. We cultured POBs with conditioned medium (CM) from Rankl-treated WT BMMs, which produce the inhibitor, and Cox2 KO BMMs, which do not. Using this model we demonstrated that PTH stimulation of (1) phosphorylation of β-catenin at Ser552/675, (2) β-catenin transcriptional activity and (3) expression of genes thought to mediate osteoblast differentiation were blocked by the Cox2/PGE\(_2\)-dependent inhibitor produced by Rankl-stimulated BMMs.

2.3 Materials and Methods

2.3.1 Materials
Bovine PTH (bPTH; 1-34) was obtained from Sigma-Aldrich (St. Louis, MO). Forskolin (cAMP agonist), H-89 (PKA inhibitor) and GF109203X (PKC inhibitor) were obtained from Enzo Life Sciences (Farmingdale, NY). Human recombinant SAA (Apo-SAA), which corresponds to human Apo-SAA1α, except for the presence of an N-terminal methionine and two substituted residues present in Apo-SAA2β, was purchased from PeproTech (Rocky Hill, NJ). Antibody for Actin C-11 (sc-1615) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-β-catenin Ser552 (#9566), phospho-β-catenin Ser675 (#9567), phospho-β-catenin Ser33/37/Thr41 (#9561), and amino terminal β-catenin (#9581) were obtained from Cell Signaling Technology (Danvers, MA).

2.3.2 Animals

Mice that produce non-functional Cox2 protein, due to disruption of Ptgs2 in a C57BL/6, 129SV background, which we call Cox2 KO mice, were the gift of Scott Morham (30). Cox2 KO mice were backcrossed into the outbred CD-1 background (31). Following 20 generations of backcrossing, the Cox2 KO mice no longer developed either renal failure or female infertility (31). Maintenance colonies heterozygous for the Cox2 gene disruption were refreshed twice a year with WT mice from Jackson Laboratory (Bar Harbor, ME) to prevent genetic drift. Mice are genotyped as described previously, and experimental mice are bred by WT x WT or KO x KO mating (31). Animal studies were performed in compliance with protocols approved by the Animal Care and Use Committee of UConn Health.
2.3.3 Cell Culture

All cell cultures were grown in humidified incubation conditions of 5% CO₂ at 37°C. Basic medium was 10% heat inactivated fetal calf serum (HIFCS), 100 U/mL penicillin, and 50 µg/mL streptomycin in α-MEM (Invitrogen, Carlsbad, CA). Osteoblast differentiation medium was basic media supplemented with 50 µg/mL phosphoascorbate. Treatment vehicles were the following: 0.001 N hydrochloric acid-acidified 0.1% bovine serum albumin (BSA) in 1x phosphate buffered saline (PBS) for PTH; 0.1% BSA in 1x PBS for osteoprotegerin (OPG), macrophage colony stimulating factor (M-CSF), and receptor activator of nuclear factor κ-B ligand (Rankl); and dimethyl sulfoxide (DMSO) for isobutyl methyl xanthine (IBMX), H-89, GF109203X, and forskolin.

2.3.4 Primary Osteoblasts (POBs)

POBs were harvested from calvariae of neonatal mice. Sutures were removed and the calvariae were minced, washed multiple times with 1x PBS, and subsequently digested with 0.5 mg/mL collagenase P (Roche Diagnostics, Indianapolis, IN) solubilized in 1 mL trypsin/EDTA and 4 mL PBS at 37°C. Four 10 minute digests were performed followed by a fifth and final digest for 90 minutes. After each digest the reaction was halted by the addition of 10% HIFCS. Cells from digests 2-5 were collected, filtered through a Nitex membrane (Millipore, Bedford, MA), and plated at a density of 50,000 cells/well in 6-well cell culture plates in differentiation medium. Medium was changed every three days. We used only freshly plated cells for all experiments.
2.3.5 Culture of Conditioned Medium (CM) with POBs

CM was collected from BMM cultures, centrifuged for 5 minutes at 800 rpm at 4°C to remove debris and frozen for later use. BMMs were cultured following the Faccio protocol: http://www.orthoresearch.wustl.edu/content/Laboratories/2978/Roberta-Faccio/Faccio-Lab/Protocols.aspx. BMMs were obtained from 8 week old mice. In brief, nucleated bone marrow cells were plated in 150 mm petri dishes (Fisher Scientific, Pittsburgh, PA) at a density of 1 x 10^7 cells/dish in basic media, supplemented with 100 ng/mL M-CSF. Cultures were expanded twice for three days each. Following expansion, BMMs were re-plated in 12-well cell culture plates at a density of 6 x 10^4 cells/well in basic media and treated with M-CSF and Rankl (both at 30 ng/mL). CM was collected after 3 days of culture, a day before tartrate resistant acid phosphatase positive multinucleated cells formed in the BMM cultures. CM was added to Cox2 KO POBs 2 hours before agonist treatment at a concentration of 3 parts CM to 1 part differentiation media. The only exception was for the study of differentiation, where treatments with agonists and CM were begun at the time of plating and continued for the entire 14 day period. Unless noted otherwise, all cultures were treated with 50 ng/mL OPG to prevent Rankl in the CM or PTH-stimulated Rankl in the POBs from inducing any remaining hematopoietic cells in the POB cultures from becoming osteoclasts and making more of the inhibitory factor (70).

2.3.6 Intracellular cAMP Measurement

On day five of culture, Cox2 KO POBs were treated with 3 parts WT or KO CM and 1 part differentiation media for 2 hours, followed by 0.5 mM IBMX for 45 minutes, and
PTH (10 nM) and FSK (10 µM) for 15 minutes. For extraction, 400 µL of ice-cold ethanol was added to each well and the cultures were detached from the plate by scraping and collected in 1.5 mL centrifuge tubes. Samples were then centrifuged at 1500 x g for 10 minutes at 4°C. The supernatant was collected and lyophilized and cAMP concentration was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

2.3.7 Western Blotting

Proteins were extracted from culture by the addition of lysis buffer (2% SDS, 10% glycerol, 62 mM Tris, pH: 6.85) and quantified using the BCA protein assay kit (Pierce, Rockford, IL). 10% SDS –PAGE was used to separate 15 µg of total protein, prior to transfer onto a nitrocellulose membrane. Membranes were washed with 1x Tris-buffered saline (TBS, pH: 7.6), blocked in blocking buffer (0.1% Tween-20, 5% (w/v) non-fat dry milk, 1x TBS), and incubated overnight at 4°C in blocking buffer supplemented with a primary antibody at the manufacturer’s suggested concentration. Membranes were subsequently washed in 1x TBS supplemented with 0.1% Tween-20 (TBST), incubated with HRP-conjugated secondary antibody, washed once more in 1x TBST, and developed using the LumiGLO chemiluminescence reagent (Cell Signaling, Danvers, MA). Densitometry was performed using ImageJ. Images of scanned films were converted into histogram form via the Analyze>Gels tool. Area under the curve was measured and normalized to the area under the corresponding β-actin curve.

2.3.8 TCF/LEF Luciferase Reporter Assay
Cox2 KO POBs were plated at 10⁵ cells/well in differentiation medium in 12 well dishes. After 24 hours they were transduced with lentiviral particles using Cignal Lenti TCF/LEF luciferase reporter (Qiagen, Valencia, CA) and Cignal Lenti Renilla luciferase control kits. Twenty four hours after transduction, medium was changed to differentiation medium. Forty eight hours after transduction, cells were treated with recombinant human SAA (10 μg/mL) or WT CM or KO CM 1 hour prior to treatment with vehicle or PTH (10 nM) for 6 hours. Dual Luciferase assay was performed using Lumat LB 9507 (Berthold Technologies, Oak Ridge, TN). Promoter activity values are expressed as arbitrary units after normalization to the Renilla reporter activity. Experiments were done in triplicate for each group.

2.3.9 Real-time (quantitative) PCR Analysis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol: 2-5 µg of total RNA was DNase treated (Ambion, Inc., Austin, TX) and subsequently converted to cDNA using the high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in 96-well plates using the Assays-on-Demand Gene Expression TaqMan primers (Applied Biosystems, Foster City, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene. Samples were amplified in duplicate and primers were checked for equal efficiency amplification over a range of target gene concentrations. The Applied Biosystems ABI Prism 7300 Sequence detection instrument was used to amplify a PCR reaction mixture comprised of 2x TaqMan Universal PCR Master Mix, 20x Assays-on-Demand Gene Expression Assay Mix and
50 ng of cDNA in a total volume of 20 µL/well at universal thermal cycling parameters. Data analysis was performed using either comparative CT (ΔΔCT) or relative standard curve methods.

2.3.10 Statistics

All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism for Microsoft Windows, Version 5.04. To compare results from experiments involving two independent variables, we used two-way ANOVA, followed by the Bonferroni pairwise multiple comparison post-test. If data were not normally distributed, a log10 transform was performed prior to ANOVA.

2.4. Results

2.4.1 WT CM abrogated PTH-stimulated cAMP production

We observed a Cox2-dependent inhibition of PTH’s osteogenic and anabolic actions both in vitro and in vivo\(^{(69, 70)}\). In cultures of bone marrow stromal cells (BMSCs), which contain both mesenchymal and hematopoietic lineages, PTH only stimulated osteoblast differentiation if Cox2-produced PGE\(_2\) was absent or if the interaction of Rankl with its receptor Rank was blocked by OPG\(^{(70)}\). We also showed that the combination of Cox2-produced PGE\(_2\) and Rankl caused BMMs to secrete a factor that inhibited PTH-stimulated osteoblast differentiation\(^{(70)}\). The required PGE\(_2\) could be produced either by the PTH induction of Cox2 in osteoblasts or by the Rankl induction of Cox2 in BMMs\(^{(70)}\). By co-culturing CM from Rankl-treated WT BMMs with POBs, we can model the inhibitory effect seen in PTH-treated BMSC cultures. We used
POBs from Cox2 KO mice to eliminate the possibility of inducing additional Cox2/PGE₂ in POBs with PTH or serum, which might affect osteoblastic differentiation. Even though POBs are washed multiple times during their collection, there can still be some hematopoietic cells remaining that can differentiate into osteoclast-like cells \(^{(70)}\). Hence, we treated the POBs with OPG to block Rankl in the CM or Rankl induced by PTH in the POBs from interacting with its receptor Rank on hematopoietic cells. We have previously shown that OPG does not affect PTH-stimulated osteoblast differentiation \(^{(70)}\).

In Figure 2A we compare the effects of no CM, WT CM and Cox2 KO CM on PTH-stimulated differentiation as measured by alkaline phosphatase (Alp) mRNA expression at 14 days of culture. Consistent with previous results, there was no difference in Alp expression among groups of vehicle-treated POBs. PTH-stimulated Alp expression was inhibited by WT CM but not by normal differentiation medium or Cox2 KO CM. Because there was no difference in effects between normal differentiation medium and Cox2 KO CM, we only compared WT and KO CM in future experiments. PTH also stimulated expression of Runx2, an early marker of osteoblast differentiation, only in the presence of Cox2 KO CM (Fig. 2B). Because PTH has much of its downstream signaling via the cAMP/PKA pathway \(^{(12, 53, 90, 91)}\), we examined the effect of WT CM on cAMP produced in response to PTH and to forskolin, a direct adenylyl cyclase (AC) agonist (Fig. 2C). Forskolin and PTH markedly stimulated cAMP production in the presence of Cox2 KO CM. WT CM blocked the stimulation of cAMP by both the PTH and forskolin. Similarly, PTH stimulated the expression of c-Fos, an early response gene mediated by the cAMP pathway \(^{(92)}\), in POBs treated with KO, not WT, CM (Fig. 2D). Thus, WT CM blocked PTH-stimulated cAMP production and signaling.
2.4.2 WT CM blocked PTH phosphorylation of β-catenin

Previous studies identified a cAMP/PKA-dependent pathway that stimulates β-catenin signaling (36, 37, 89). The hallmark of this pathway is the phosphorylation of β-catenin at Ser552 and/or Ser675 by PKA. We compared the effects of WT and Cox2 KO CM on PTH-stimulated phosphorylation at these two sites. PTH stimulated phosphorylation at both sites only in the presence of KO CM (Fig 3A). Phosphorylation was observed as early as 15 minutes of treatment and was sustained at 45 minutes of treatment (Fig. 3A). PTH caused a 3- and 2-fold increase of Ser552 and Ser675 phosphorylation, respectively, at 15 min (Fig. 3B-C), but did not increase total β-catenin levels (Fig. 3D).

To determine if this effect was dependent on PKA, we treated with H89, a PKA inhibitor (Fig. 4). H89 had no effect on POBs treated with WT CM. In POBs co-cultured with KO CM, the PTH-stimulated increase in Ser552 and Ser675 phosphorylation was abrogated by the presence of H89 (Fig. 4A). Although H89 is a selective inhibitor of PKA, it has been reported to have off-target effects on PKC (93). In order to rule out involvement of PKC, we treated with a specific PKC inhibitor, GF109203X, at a dose previously shown to inhibit the effects of phorbol myristate acetate (94). GF109203X had no effect on PTH stimulation of β-catenin phosphorylation (Fig. 4B).

To examine whether or not β-catenin destruction was being affected by PTH treatment we examined both total levels of β-catenin as well as the GSK3β-stimulated phosphorylation sites that mark β-catenin for ubiquitination and destruction.
(Ser33/37/Thr41). There was no effect of PTH on levels of total β-catenin or β-catenin marked for destruction in the presence of KO CM (Fig. 4E-F).

2.4.3 WT CM or SAA blocked PTH stimulated β-catenin transcriptional activity

β-catenin signals by binding to TCF/LEF transcription factors in the nucleus, which leads to the transcription and expression of Wnt-responsive genes \(^{(95)}\). Although previous reports have differed on whether or not phosphorylation of β-catenin at Ser552 and Ser675 results in an increase in stability and nuclear translocation, they have generally reported increased transcriptional activity of β-catenin \(^{(36, 37, 41, 89)}\). To examine effects on transcriptional activity in POBs, we measured activity of a TCF/LEF-inducible firefly luciferase reporter normalized to activity of a constitutively active renilla luciferase construct. We compared the effects on PTH-stimulated TCF/LEF activity of WT CM and SAA, a human analog of Saa3, which we have proposed to be the specific factor in the WT CM that inhibits the effects of PTH \(^{(71)}\).

LiCl, which can activate Wnt signaling by inhibiting GSK3β, stimulated luciferase activity that was not inhibited by SAA (Fig. 5A). In the experiments shown in Fig. 5B-C, 6 hours of treatment with PTH induced a 4- to 5-fold increase in TCF/LEF activity in POBs treated with no CM or with Cox2 KO CM. Both WT CM and SAA abrogated the PTH stimulation of luciferase activity. Hence, WT CM and SAA have similar inhibitory effects. These data suggest that SAA does not inhibit a Wnt signaling pathway acting via GSK3β but does inhibit the PTH stimulation of β-catenin activity mediated by phosphorylation of β-catenin at Ser552 and Ser675.
2.4.4 WT CM blocked the PTH regulation of genes involved in Wnt signaling

To examine effects of the inhibitory CM on the PTH regulation of specific genes known to be involved in Wnt signaling, we treated POBs for 3 hours with PTH and compared responses in WT or Cox2 KO CM. Several Wnts—*Wnt4*, *Wnt7b*, and *Wnt10b*—were increased in expression only in KO CM treated groups (Fig. 6). *Wnt4* has been associated with the anabolic effects of PTH and found to be stimulatory for osteoblastogenesis and bone formation \(^{(85, 96)}\). Expression of *Wnt7b* can be induced by PTH, has been shown to increase with osteoblastic differentiation and to increase bone mass as well as osteoblast number *in vivo* \(^{(97)}\). *Wnt10b* is an important factor in coupling the actions of osteoblasts and osteoclasts, while also being able to enhance commitment to the osteoblastic lineage \(^{(98, 99)}\). In contrast, the expression of *Wnt5a*, reported to antagonize canonical Wnt signaling by stimulating destruction of β-catenin via GSK3β \(^{(100)}\), was unaffected by PTH treatment in both WT and KO CM treated groups (Fig. 6). We were unable to detect *Wnt3A* mRNA (data not shown). Expression of *Nfatc1*, shown to be important for non-canonical Wnt signaling \(^{(101)}\) and for the PTH induction of *Cox2* \(^{(102)}\), was markedly induced by PTH only in KO CM. Expression of c-Myc, a Wnt target gene in colorectal cancer \(^{(103)}\), was also increased by PTH only in KO CM.

In contrast to genes that positively regulate Wnt signaling, the expression of genes thought to inhibit Wnt signaling was inhibited by PTH in Cox2 KO CM (Fig. 6). Genes coding for the canonical Wnt signaling inhibitors Sfrp1 and Dkk1, which inhibit Wnt agonist interaction with Frizzled and Lrp5/6, respectively, were undetectable in cultures treated with PTH in the presence of KO CM. Hence, the inhibitory CM blocked the PTH
stimulation of multiple genes that may enhance osteogenic or anabolic responses and
the PTH inhibition of several genes that inhibit these Wnt signaling.

2.5. Discussion

Our results suggest a prominent role for the β-catenin sites Ser552 and Ser675 in
mediating PTH-stimulated β-catenin transcriptional activity and Wnt-related gene
expression. We demonstrated that a novel inhibitor, produced by Cox2-expressing
BMMs and shown to block PTH-stimulated osteoblast differentiation in vitro (70), blocked
PTH-stimulated cAMP production and PKA phosphorylation at these sites, along with
subsequent β-catenin transcriptional activity. Although PTH can activate both PKA and
PKC signaling pathways, our observations strongly suggest that the direct effects of
PTH on β-catenin signaling and osteoblast differentiation occur via Gαs/cAMP-initiated
pathways as previously reported (12,85).

A number of studies have reported that PTH promotes increased β-catenin
transcriptional activity but the specific steps by which this occurs are still not clear. PTH
has been shown to be a potent inhibitor of canonical Wnt antagonists, including Dkk1,
Sost, and Sfrp, which can lead to enhanced Wnt signaling and stabilization of β-catenin
(14, 104-106), and to increase the expression of Wnt agonists (107). An early study in rat
osteosarcoma UMR-106 cells reported that PTH acted via the canonical Wnt signaling
pathway to increase Lrp6 and Fzd-1 expression, decrease Dkk1 expression, and
increase β-catenin levels and activity and that these effects were dependent on cAMP
signaling (39). However, PTH was also reported to activate β-catenin signaling by
directly recruiting dishevelled independently of Wnt or Lrp5/6 in UMR cells (16) and by

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directly inactivating GSK-3β in human osteosarcoma Saos-2 cells \(^{(18)}\). Another study in UMR cells and \textit{in vivo} showed that PTH stabilized β-catenin in osteoblasts by activating binding of its receptor PTH1R to Lrp6 and by activating PKA to phosphorylate Lrp6 \(^{(37, 41, 47)}\). One other group has linked PTH to phosphorylation of Ser675 on β-catenin \(^{(89)}\). They used bone marrow stromal cells and MC3T3-E1 cells and found that PTH stimulated increased β-catenin activity but did not increase total β-catenin levels. In our murine POB model, we did not find any evidence of GSK-3β mediated phosphorylation of β-catenin at Ser33/37/Thr41 nor did we observe any increases in total β-catenin, suggesting that PTH acted to increase the signaling efficiency of the current population of β-catenin rather than preventing its destruction. Our results agree with Taurin et al. who reported that PKA phosphorylation of β-catenin at Ser552 and Ser675 increased β-catenin transcriptional activity, while leaving the destruction markers and complex unaffected \(^{(37, 104)}\).

Our \textit{in vitro} results in POBs treated continuously with PTH are similar to our \textit{in vivo} effects in WT and Cox2 KO mice continuously infused with PTH for 12 or 21 days. Infused PTH was anabolic for bone only in Cox2 KO mice, not in WT mice. PTH infusion increased expression in tibiae of \textit{Wnt4} and \textit{Wnt10b} and decreased expression of \textit{Sfrp1}, \textit{Dkk1} and \textit{Sost} only in Cox2 KO mice. Expression of \textit{Wnt3a} was undetectable in our \textit{in vitro} studies and was not regulated by PTH in either WT or KO mice in the infusion studies. \textit{Wnt3a} is frequently used as an exogenous agent to induce canonical Wnt signaling. Our data suggest that other Wnts (\textit{Wnt4}, \textit{Wnt7b}, and \textit{Wnt10}) are more likely to be involved in the direct osteogenic/anabolic effects of PTH. One interesting possibility is that the PTH-induced phosphorylation of β-catenin at Ser552 and Ser675
could be priming osteoblasts for subsequent Wnt-signaling to take place by increasing expression of these Wnts and decreasing the expression of Wnt inhibitors.

These studies highlight the potential role of the Cox2-dependent inhibitory factor for modulating the osteogenic/anabolic actions of PTH. We have recently identified the inhibitory factor as Saa3. Saa3 can be stimulated by PTH itself. PTH induces Cox2/PGE$_2$ and Rankl in the osteoblast lineage, and Rankl then commits monocyte/macrophages to the osteoclast lineage and induces more Cox2/PGE$_2$ in preosteoclasts, leading to the secretion of Saa3 (Fig. 7). It seems likely that this inhibitory factor suppresses the anabolic responses to continuously elevated PTH and may also inhibit the anabolic responses to intermittently elevated PTH in vivo. Saa3 is an acute phase protein, generally considered to be associated with acute and chronic inflammation, and its levels can be increased many fold under inflammatory circumstances. Hence, Saa3 might be an important means by which inflammatory agents suppress PTH-stimulated anabolic actions and cause bone loss and, therefore, Saa3 might be implicated in the bone loss associated with aging and chronic inflammation.

Our results suggest that the suppression of the osteogenic/anabolic responses to PTH, associated with Cox2 expression, is due to the inhibition of PTH/cAMP/PKA stimulated β-catenin signaling by Rankl-stimulated BMMs. Thus, the osteogenic/anabolic effects of PTH might be enhanced by interrupting the production of the Cox2-mediated induction of Saa3.
Fig. 2. CM from WT BMMs inhibited PTH-stimulated osteoblast differentiation and cAMP production. (A) Cox2 KO POBs were cultured for 14 days in the presence of no CM (differentiation media), WT CM, or Cox2 KO CM and either vehicle or PTH (10 nM). On day 14 of culture, Alp mRNA expression was measured by qRT-PCR. (B) On day 5 of culture, Cox2 KO POBs were treated with vehicle or PTH (10 nM) for 3 hours. Runx2 expression was measured by qRT-PCR. (C) On day 5 of culture, Cox2 KO POBs were treated with vehicle or PTH (10 nM) for 15 minutes. cAMP was measured by ELISA. (D) On day 5 of culture, Cox2 KO POBs were treated with vehicle or PTH (10 nM) for 3 hours. c-Fos expression was measured by qRT-PCR. Bars represent mean ± SEM, n=3. a Significant effect of PTH, p<0.01. b Significant difference compared to WT CM, p<0.01.
Fig. 3. PTH increased phosphorylation of β-catenin at ser552 and ser675 in the absence of WT CM. Cox2 KO POBs were treated with vehicle or PTH (10 nM) on day 5 of culture and Western analysis was performed. (A) Time course examining effects of PTH on β-catenin phosphorylation. (B-E) Densitometry of bands was performed and normalized to β-actin. Vehicle-treated WT CM was set to 1. Bars represent means ± SEM, n=4. αSignificant effect of PTH, p<0.01; βp<0.05. γSignificant difference compared to WT CM, p<0.01; δp>0.05.
**Fig. 4. PTH phosphorylated β-catenin at ser552 and ser675 in a PKA-dependent manner.** Cox2 KO POBs were treated with vehicle or 10 nM PTH on day 5 of culture and Western analysis was performed. (A) Cultures treated with 10 nM PTH for 15 minutes following WT/KO CM and ± H89 (30 µM) pretreatment. (B) Cultures treated as noted with the addition of ± GF109203X (1 µM). (C-G) Densitometry was performed on KO CM treated groups, values were normalized to β-actin and vehicle-treated groups were set to 1. Bars represent means ± SEM, n=3. *Significant effect of PTH, p<0.05.
Fig. 5. PTH increased TCF/LEF reporter activity in the absence of WT CM. (A) Cultures were treated for 6 hours with LiCl (20 mM) or NaCl (20 mM). (B, C) Cultures were treated for 2 hours with no CM, WT CM, Cox2 KO CM, or SAA (10 μg/mL) prior to 6 hours of treatment with vehicle or PTH (10 nM). Panels (B) and (C) represent independent transductions. Promoter activity values are expressed as relative firefly luciferase units normalized to Renilla reporter activity. Bars are means ± SEM, n=3.

aSignificant difference compared to vehicle, p<0.01. bSignificant difference compared to WT CM, p<0.01. cSignificant effect compared to SAA, p<0.01.
Fig. 6. PTH regulated expression of genes associated with Wnt signaling only in the absence of WT CM. Cox2 KO POBs were treated with vehicle or 10 nM PTH for 3 hours on day 5 of culture. mRNA was measured by qRT-PCR. Genes whose expression was undetectable are marked Und. Bars are means ± SEM, n=3.

aSignificant effect of PTH, p<0.01; b p<0.05. cSignificant effect compared to WT CM, p<0.01; d p<0.05.
Fig. 7. Proposed means by which PTH inhibits its own osteogenic/anabolic actions. PTH stimulates Rankl and Cox2/PGE\(_2\) in osteoblast (OB) lineage cells. Rankl acts on osteoclast (OC) lineage cells to commit them to become osteoclasts and to stimulate more Cox2/PGE\(_2\). The combination of Rankl and PGE\(_2\) cause cells committed to become osteoclasts to secrete Saa3. In the presence of Saa3, PTH-stimulated cAMP production is abrogated, preventing subsequent PKA activation and β-catenin signaling. When PTH is unable to initiate this negative feedback pathway, either by absence of osteoclasts or Cox2, β-catenin is phosphorylated at Ser552 and Ser675 and can stimulate gene transcription associated with osteoblastic differentiation.
3. PTH Stimulation of RANKL in Primary Osteoblasts Is Independent of PTH-Stimulated cAMP.

3.1 Abstract

Parathyroid hormone (PTH) can stimulate both bone formation and resorption. When PTH is given continuously, resorption is greater than formation and bone is lost. We showed that the osteogenic actions of continuous PTH \textit{in vitro} are suppressed by a factor that blocks PTH-stimulated cAMP production in osteoblastic cells. The production of this factor is dependent on the expression of cyclooxygenase 2 (Cox2), the major enzyme regulating prostaglandin production. When we treated wild type (WT) and Cox2 knockout (KO) mice with PTH infusion for 12-21 days, we found that anabolic actions of PTH were suppressed in WT, but not KO mice. However, the PTH stimulation of bone resorption was the same in both WT and KO mice. We have identified the inhibitory factor \textit{in vitro} as serum amyloid A 3 (Saa3) and shown that Saa3 is secreted by bone marrow macrophages (BMMs) treated with RANKL. In this study we use the conditioned media (CM) from RANKL-treated WT BMMs or a recombinant, human-homolog of murine Saa3 (SAA) to study the involvement of cAMP-activated signaling in the PTH induction of \textit{Rankl}. We cultured primary osteoblasts (POBs) from neonatal calvaria to confluence (5 days), treated with PTH (10 nM) in the presence or absence of CM or SAA (10 µg/mL) for 3 h and measured gene expression by qPCR. SAA concentration was determined by dose response as measured by cAMP signaling inhibition. As expected, both CM and SAA blocked the PTH-stimulated gene expression of cAMP-regulated receptor activity modifying protein 3 (Ramp3). However, PTH stimulated \textit{Rankl} expression was not decreased by CM or SAA. The protein kinase
A (PKA) inhibitor, H-89, blocked PTH-stimulated *Ramp3* expression but had no effect on PTH-stimulated *Rankl* expression. PTH has also been shown to activate the protein kinase C (PKC) pathway, which subsequently signals via extracellular-signal-regulated kinases (ERKs). The PKC inhibitor GF109203X and the ERK inhibitor PD98059 both blocked PTH-stimulated *Rankl* expression but did not decrease PTH-stimulated *Ramp3*. The calcium chelation agent BAPTA also blocked PTH-stimulated *Rankl* expression. In conclusion, our results indicate that PTH-stimulated *Rankl* in POBs is independent of cAMP signaling and is likely to depend upon the PKC pathway. These data support our PTH infusion study, which indicated that the anabolic and catabolic effects of PTH occur via different signaling pathways.

### 3.2 Introduction

Receptor activator of nuclear kappa-b ligand (RANKL) is an important factor in bone metabolism, mediating the crosstalk between osteoblast and osteoclast populations in order to maintain homeostasis. While RANKL is expressed by a multitude of cells including dendritic and mammary epithelial cells \(^{(108)}\), its expression in the osteoblast lineage acts to stimulate the RANK receptor on osteoclast precursors to cause differentiation and activate resorption \(^{(109)}\). In order to counteract this resorption response, osteoblasts can also secrete osteoprotegerin (OPG) which acts as a decoy receptor for RANKL thereby competitively inhibiting the osteoclast response.

Parathyroid hormone (PTH) enacts its effects on resorption by stimulating RANKL expression in the osteoblast lineage \(^{(110-112)}\). Previous results have suggested that PTH acts through the cAMP/PKA signaling pathway to stimulate RANKL expression in osteoblasts \(^{(52, 53, 113)}\). Our lab has recently discovered a factor secreted by osteoclast
precursors in response to prostaglandin E2 \((\text{PGE}_2)\) that acts as a potent cAMP inhibitor in osteoblasts \((69, 70)\). This factor was identified as serum amyloid a3 \((\text{Saa3})\) \((71)\). We have developed an \textit{in vitro} model by which we can introduce Saa3 into culture via conditioned medium \((\text{CM})\) collected from cultures of bone marrow macrophages \((\text{BMMs})\) stimulated down the osteoclast lineage by treatment of RANKL and M-CSF \((70)\). In this study we use this model to determine the effects of the cAMP inhibitor, Saa3, upon PTH stimulation of RANKL in primary osteoblasts \((\text{POBs})\). By doing so we hope to determine whether cAMP signaling is required for PTH-stimulation of RANKL.

### 3.3 Materials and Methods

#### 3.3.1 Materials

Bovine PTH \((\text{bPTH}; 1\text{-}34)\) was obtained from Sigma-Aldrich \((\text{St. Louis, MO})\). \text{PGE}_2 was obtained from Cayman Chemical Company \((\text{Ann Arbor, MI})\). FSK \((\text{cAMP agonist})\), H-89 \((\text{PKA inhibitor})\), GF109203X \((\text{PKC inhibitor})\), PD98059 \((\text{MEK/ERK inhibitor})\), and BAPTA \((\text{Calcium chelation agent})\) were obtained from Enzo Life Sciences \((\text{Farmingdale, NY})\). PD 0325901 \((\text{MEK/ERK inhibitor})\) was obtained from Sigma-Aldrich \((\text{St. Louis, MO})\). Human recombinant SAA \((\text{Apo-SAA})\), which corresponds to human Apo-SAA1α, except for the presence of an N-terminal methionine and two substituted residues present in Apo-SAA2β, was purchased from PeproTech \((\text{Rocky Hill, NJ})\). Antibody for Actin C-11 \((\text{sc-1615})\) was obtained from Santa Cruz Biotechnology \((\text{Santa Cruz, CA})\). Total MEK \((9122)\), total ERK \((9107)\), p-MEK \((9121)\), and p-ERK \((9101)\) antibodies were obtained from Cell Signaling Technology \((\text{Danvers, MA})\).

#### 3.3.2 Animals
Mice that produce non-functional Cox2 protein, due to disruption of \textit{Ptgs2} in a C57BL/6, 129SV background, which we call Cox2 KO mice, were the gift of Scott Morham \cite{30}. Cox2 KO mice were backcrossed into the outbred CD-1 background \cite{31}. Following 20 generations of backcrossing, the Cox2 KO mice no longer developed either renal failure or female infertility \cite{31}. Maintenance colonies heterozygous for the Cox2 gene disruption were refreshed twice a year with WT mice from Jackson Laboratory (Bar Harbor, ME) to prevent genetic drift. Mice are genotyped as described previously, and experimental mice are bred by WT x WT or KO x KO mating \cite{31}. Animal studies were performed in compliance with protocols approved by the Animal Care and Use Committee of UConn Health.

\subsection*{3.3.3 Cell Culture}

All cell cultures were grown in humidified incubation conditions of 5\% CO\textsubscript{2} at 37\textdegree{}C. Basic medium was 10\% heat inactivated fetal calf serum (HIFCS), 100 U/mL penicillin, and 50 \mu g/mL streptomycin in \alpha-MEM (Invitrogen, Carlsbad, CA). Osteoblast differentiation medium was basic media supplemented with 50 \mu g/mL phosphoascorbate. Treatment vehicles were the following: 0.001 N hydrochloric acid-acidified 0.1\% bovine serum albumin (BSA) in 1x phosphate buffered saline (PBS) for PTH; 0.1\% BSA in 1x PBS for osteoprotegerin (OPG), macrophage colony stimulating factor (M-CSF), and receptor activator of nuclear factor \kappa-B ligand (Rankl); and dimethyl sulfoxide (DMSO) for isobutyl methyl xanthine (IBMX), H-89, GF109203X, and forskolin.

\subsection*{3.3.4 Primary Osteoblasts (POBs)
POBs were harvested from calvariae of neonatal mice. Sutures were removed and the calvariae were minced, washed multiple times with 1x PBS, and subsequently digested with 0.5 mg/mL collagenase P (Roche Diagnostics, Indianapolis, IN) solubilized in 1 mL trypsin/EDTA and 4 mL PBS at 37°C. Four 10 minute digests were performed followed by a fifth and final digest for 90 minutes. After each digest the reaction was halted by the addition of 10% HIFCS. Cells from digests 2-5 were collected, filtered through a Nitex membrane (Millipore, Bedford, MA), and plated at a density of 50,000 cells/well in 6-well cell culture plates in differentiation medium. Medium was changed every three days. We used only freshly plated cells for all experiments.

3.3.5 Culture of Conditioned Medium (CM) with POBs

CM was collected from BMM cultures, centrifuged for 5 minutes at 800 rpm at 4°C to remove debris and frozen for later use. BMMs were cultured following the Faccio protocol: http://www.orthoresearch.wustl.edu/content/Laboratories/2978/Roberta-Faccio/Faccio-Lab/Protocols.aspx. BMMs were obtained from 8 week old mice. In brief, nucleated bone marrow cells were plated in 150 mm petri dishes (Fisher Scientific, Pittsburgh, PA) at a density of 1 x 10^7 cells/dish in basic media, supplemented with 100 ng/mL M-CSF. Cultures were expanded twice for three days each. Following expansion, BMMs were re-plated in 12-well cell culture plates at a density of 6 x 10^4 cells/well in basic media and treated with M-CSF and Rankl (both at 30 ng/mL). CM was collected after 3 days of culture, a day before tartrate resistant acid phosphatase positive multinucleated cells formed in the BMM cultures. CM was added to Cox2 KO
POBs 2 hours before agonist treatment at a concentration of 3 parts CM to 1 part differentiation media. The only exception was for the study of differentiation, where treatments with agonists and CM were begun at the time of plating and continued for the entire 14 day period. Unless noted otherwise, all cultures were treated with 50 ng/mL OPG to prevent Rankl in the CM or PTH-stimulated Rankl in the POBs from inducing any remaining hematopoietic cells in the POB cultures from becoming osteoclasts and making more of the inhibitory factor \(^{70}\).

### 3.3.6 Real-time (quantitative) PCR Analysis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol: 2-5 µg of total RNA was DNase treated (Ambion, Inc., Austin, TX) and subsequently converted to cDNA using the high capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed in 96-well plates using the Assays-on-Demand Gene Expression TaqMan primers (Applied Biosystems, Foster City, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene. Samples were amplified in duplicate and primers were checked for equal efficiency amplification over a range of target gene concentrations. The Applied Biosystems ABI Prism 7300 Sequence detection instrument was used to amplify a PCR reaction mixture comprised of 2x TaqMan Universal PCR Master Mix, 20x Assays-on-Demand Gene Expression Assay Mix and 50 ng of cDNA in a total volume of 20 µL/well at universal thermal cycling parameters. Data analysis was performed using either comparative CT (ΔΔCT) or relative standard curve methods.

### 3.3.7 Western Blotting
Proteins were extracted from culture by the addition of lysis buffer (2% SDS, 10% glycerol, 62 mM Tris, pH: 6.85) and quantified using the BCA protein assay kit (Pierce, Rockford, IL). 10% SDS –PAGE was used to separate 15 µg of total protein, prior to transfer onto a nitrocellulose membrane. Membranes were washed with 1x Tris-buffered saline (TBS, pH: 7.6), blocked in blocking buffer (0.1% Tween-20, 5% (w/v) non-fat dry milk, 1x TBS), and incubated overnight at 4°C in blocking buffer supplemented with a primary antibody at the manufacturer’s suggested concentration. Membranes were subsequently washed in 1x TBS supplemented with 0.1% Tween-20 (TBST), incubated with HRP-conjugated secondary antibody, washed once more in 1x TBST, and developed using the LumiGLO chemiluminescence reagent (Cell Signaling, Danvers, MA). Densitometry was performed using ImageJ. Images of scanned films were converted into histogram form via the Analyze>Gels tool. Area under the curve was measured and normalized to the area under the corresponding β-actin curve.

3.3.8 Statistics

All data are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism for Microsoft Windows, Version 5.04. To compare results from experiments involving two independent variables, we used two-way ANOVA, followed by the Bonferroni pairwise multiple comparison post-test. If data were not normally distributed, a log10 transform was performed prior to ANOVA.

3.4 Results

3.4.1 PTH stimulates RANKL in POBs in the absence of cAMP signaling

In order to determine the effects of Saa3 on PTH stimulation of RANKL expression we examined both RANKL and Ramp3 mRNA expression. Ramp3 is a cAMP response gene used to indirectly measure levels of cAMP/PKA signaling. We
observed that in cultures pretreated with either wild type conditioned media (WT CM) (Fig. 8a) or exogenous recombinant human serum amyloid a (SAA) (Fig. 8b) that PTH was unable to stimulate cAMP signaling as measured by Ramp3 expression. Unexpectedly, we found that despite the inability of PTH to stimulate cAMP signaling in the presence of SAA, PTH was able to stimulate a robust RANKL response comparable to that seen in the SAA-free group (Fig. 8c).

### 3.4.2 PTH stimulates RANKL expression through the PKC pathway

In light of our results suggesting that PTH acts to increase RANKL expression independently of cAMP/PKA signaling, we then began investigating other pathways that may be involved. While PTH is known to signal through its G-protein coupled receptor to stimulate PKA signaling through the production of cAMP via Gαs stimulation of adenylate cyclase, it can also signal through PKC. Current thought is that PTH acts through Gαq to activate phospholipase C, causing the production of inositol triphosphate (IP3) and subsequent Ca2+ mobilization to activate PKC (11, 12). It is thought that once activated, PKC acts through the MEK/ERK pathway to affect gene transcription (60). Other studies have also suggested that, while not directly linked to PTH, the PKC agonist, PMA, and the ionophore, ionomycin, can both stimulate RANKL expression (58). Considering these findings regarding the PKC pathway and our own data suggesting that PTH may act independently of the cAMP/PKA pathway to stimulate RANKL expression, we challenged our system with a battery of inhibitors in order to dissect the role of Ca2+, PKC, and ERK in this process. We found that PTH was unable to stimulate RANKL mRNA expression in the presence of the PKC inhibitor, GF109203X,
the ERK inhibitor, PD98059, and the calcium chelation agent, BAPTA (Fig. 9a). However, RANKL expression remained intact as compared to vehicle when our system was challenged with the PKA inhibitor, H89 (Fig. 9a). In order to confirm that this inhibition occurred in the presence of robust cAMP signaling, we observed Ramp3 expression and only found its inhibition in the H89 pretreated group (Fig. 9b). This complements our previous results where PTH was able to stimulate RANKL in the absence of cAMP/PKA signaling, we now see that PTH’s ability to stimulate RANKL expression can be inhibited despite robust cAMP/PKA signaling. Recent findings have suggested that PD98059 has off-target effects on intracellular Ca\(^{2+}\)\(^\text{(114)}\). To address this possibility we repeated our experiment with the inclusion of another inhibitor of MEK/ERK signaling, PD325901, which does not exhibit these off-target effects. Our results show that both inhibitors of MEK/ERK signaling shut down PTH stimulation of RANKL expression, confirming the involvement of the MEK/ERK portion of the pathway (Fig. 9c).

It was then of interest to confirm our mRNA results with western blot. We examined the phosphorylation states of MEK and ERK to determine activation of the pathway in response to PTH, in the presence of either WT CM or KO CM. In the presence of WT CM, PTH was unable to increase phosphorylation of either MEK or ERK (Fig. 10a). Further, total levels of both proteins appeared to be decreased in response to PTH in the WT CM pretreated groups (Fig. 10a). No effect of PTH upon either the simulation or inhibition or ERK signaling was evident in KO CM pretreated cultures (Fig. 10a). Similarly, neither an increase nor decrease of MEK/ERK signaling was observed following out to three hours of PTH treatment (Fig. 10b).
3.5 Discussion

Our results represent the first to link PTH-stimulated PKC signaling to RANKL gene expression in primary osteoblasts. We hypothesize from our results that PTH activates PKC via increases in intracellular Ca\(^{2+}\) and causes subsequent MEK/ERK signaling to stimulate RANKL gene expression. Although our western blot results were inconclusive regarding MEK/ERK stimulation by PTH, these results cannot speak to a change in localization of MEK/ERK or other more nuanced effects. Future studies would do well to perform dose response experiments with the signaling inhibitors used herein to confirm the level of involvement of each Ca\(^{2+}\), PKC, and MEK/ERK.

Additionally, the MEK/ERK signal may be more transient than our experiments were designed to detect and shorter time points may need to be investigated.

Apart from our results, contemporaneous studies have implicated the actions of PKC to stimulate RANKL induction of osteoclastogenesis. Specifically, RANKL-stimulated PKC\(\beta\) can inhibit GSK3\(\beta\) to allow for NFATc1 translocation to the nucleus to support differentiation and activation of osteoclasts from cultures of BMMs \(^{(68)}\).

However, our results showing that RANKL induction is dependent on PKC signaling may shine new light and urge for caution when manipulating the PKC pathway \textit{in vivo} or in mixed \textit{in vitro} culture. Then a compounding effect may not be considered whereby the PKC inhibitor prevents PKC from stimulating RANKL expression by osteoblasts and also prevents RANKL stimulation of PKC mediated osteoclastogenesis within osteoclasts.

Inhibition of the PKC pathway may then represent a novel target for treating bone diseases whose basis lies in overactive resorption. Our data suggest that the inclusion
of a PKC inhibitor alongside continuous PTH treatment not only proves an interesting study but one that may prevent the deleterious catabolic effects associated with continuous infusion.
Fig. 8. Inhibition of cAMP signaling does not prevent PTH stimulation of RANKL expression. On day 5 of culture, Cox-2 KO POBs were treated with vehicle or PTH (10 nM) for 3 hours following 2 hour pretreatment with WT or KO CM or Saa. mRNA was then isolated and qRT-PCR was performed. Bars represent mean ± SEM, n=3.

aSignificant effect compared to WT CM or SAA, p<0.01. bSignificant effect of PTH, p<0.01
Fig. 9. PTH stimulation of RANKL is dependent on calcium/PKC/ERK signaling.
On day 5 of culture, WT POBs were treated with vehicle or PTH (10 nM) for 3 hours following 2 hour pretreatment with H89: 30 μM, GF109203X: 0.6 μM, PD98059: 50 μM, PD0325901: 50 μM or BAPTA 50 μM. mRNA was then isolated and qRT-PCR was performed. Bars represent mean ± SEM, n=3. aSignificant effect of PTH, p<0.01.
Fig. 10. PTH decreases MEK/ERK in the presence of WT CM (A), and does not increase ERK signaling in basal conditions (B). (A) On day 5 of culture, COX-2 KO POBs were treated with vehicle or PTH (10 nM) for 15 minutes (if not indicated) following 2 hour pretreatment with WT or KO CM and 45 minute pretreatment of the following: H89: 30 μM, or GF: 0.6 μM. (B) On day 5 of culture, COX-2 KO POBs were treated with vehicle or PTH (10 nM) for 0, 1, 2, or 3 hours. Protein was collected and western blot was performed.
4. Summary and Future Directions

In this study we showed that in the absence of Saa3, PTH stimulates phosphorylation of β-catenin at serines 552 and 675 which cause an increase in transcriptional activation of TCF/LEF and subsequent expression of Wnt target genes. Further, we observed that PTH can stimulate expression of RANKL independent of cAMP/PKA signaling in osteoblasts. The alternative pathway by which PTH causes RANKL expression in these cultures seems to be through Ca\textsuperscript{2+}/PKC. Together these results point to a model by which PTH acts anabolically on bone metabolism via cAMP/PKA and catabolically via Ca\textsuperscript{2+}/PKC. However, due to the Saa3 negative feedback loop the anabolic effects, specifically those affecting phosphorylation of β-catenin, are muted in basal conditions. Although not exhaustive, our results posit no parallel negative feedback loop by which PTH can silence its own catabolic effects. These observations help explain why PTH acts largely catabolically in vivo.

Still to be determined is the contribution of this PTH/β-catenin pathway to osteoblast differentiation and bone formation in general. First steps to quantify this contribution may include determining whether or not PTH stimulation of osteoblasts in the absence of Saa3 primes subsequent Wnt signaling. Our results indicate that several Wnt agonists experience a rapid upregulation in gene expression, while Wnt antagonists experience a downregulation in response to PTH treatment in the absence of Saa3. Observations at later time points may reveal the downstream effects of this gene expression or indeed if cultures are more sensitive to exogenous Wnt signaling post PTH treatment. In order to determine the importance of phosphorylation of serines 552 and 675 in β-catenin signaling, and bone biology as a whole, the incorporation of
alanine substitutions at these sites in an in vivo model would be of interest. Previous in vitro studies have shown that ser-to-ala substitutions at these sites abolishes the ability of PKA to phosphorylate these sites \(^{(37)}\). Observing how mutations at these sites affect not only both the β-catenin and Rankl pathways \textit{in vitro} and further how they affect bone metabolism \textit{in vivo} is of great interest.
5. References


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