

**PROTEOGLYCAN 4: A DYNAMIC REGULATOR OF PARATHYROID
HORMONE BIOLOGIC ACTIONS**

by

Chad M. Novince

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Oral Health Sciences)
in The University of Michigan
2011

Doctoral Committee:

Professor Laurie K. McCauley, Chair
Professor Jan C. Hu
Professor Russell S. Taichman
Associate Professor Hedwig S. Murphy

DEDICATION

To my parents, Loretta and William

To my bothers, Ryan and Zachary

ACKNOWLEDGEMENTS

I express sincere gratitude to all parties that provided me support throughout my doctoral studies in the Oral Health Sciences Ph.D. program. I am deeply appreciative of the time and effort that Dr. Laurie McCauley, my Ph.D. mentor and the chair of my dissertation committee, devoted to my scientific training. I am thankful for her invaluable guidance and support, which empowered me to have a successful Ph.D. training experience. Her sincere passion for the advancement of science and commitment to mentoring is inspiring to developing scientist.

I acknowledge and thank the members of my dissertation committee; Dr. Laurie McCauley, Dr. Russell Taichman, Dr. Jan Hu, and Dr. Hedwig Murphy for providing me with the direction and support that enabled me to make continual progress throughout my doctoral studies. I am grateful to the members of my committee for unselfishly sharing their expertise and individually meeting with me on a regular basis during my Ph.D. training. The enthusiasm and encouragement conveyed by my committee members towards my doctoral research and training was deeply motivational.

I individually acknowledge Dr. Russell Taichman and Dr. Thomas Rosol for their informal mentorship. Scientific interactions with Drs. Taichman and Rosol were formative experiences during my doctoral training. Moreover, their willingness to

collaborate on scientific projects broadened the scope and impact of my Ph.D research studies.

I am ever indebted to the members of Dr. Laurie McCauley's research laboratory for sharing their vast knowledge and expertise in carrying out scientific research. The enthusiasm for research and discovery that is conveyed by the McCauley lab members provided for an exciting and motivational Ph.D. training experience. I thank Amy Koh, Jan Berry, Glenda Pettway, Xin Li, Jinhui Liao, Flavia Pirih, Serkin Park, Zhengyan Wang, Sun Wook Cho, Fabiana Soki, Megan Michalski, Matt Eber, Payam Entezami, Mallory Van Putten, Naomi Lin, and David Sadler for their friendship and support during my doctoral research training. Individually, I extend sincere thanks to Amy, Megan, and Payam for playing an integral role in the progress and success of my Ph.D. research studies. Other individuals I acknowledge for their contributions include Junro Yamashita, Julie Marchesan, Chris Wilson, Ben Sinder, Ken Kozloff, Chris Strayhorn, Chris Jung, Taocong Jin, Holly Brown, Ramiro Toribio, Thomas Wronski, and Matthew Warman.

Special thanks go to Dr. Charlotte Mistretta, director of the Oral Health Sciences Ph.D. program, for her sincere commitment to the well being and success of the Ph.D. students. Her guidance and enthusiastic support throughout the Ph.D. training experience was a source of inspiration that was very meaningful to me. I thank Dr. Robert Bradley for his pre-candidate mentorship. I am grateful to Dean Peter Polverini for taking a sincere interest in my Ph.D. training and professional development. I also extend thanks to Dr. Darnell Kaigler for his positive encouragement and generous advice.

I acknowledge and thank Patricia Schultz, Janet Riggs, and Diane Lafferty for their guidance and assistance in applying for grants and managing the administrative aspects of my Ph.D. training.

Concerning the clinical component of my dual D.D.S./Ph.D. studies I recognize and thank Dr. Marilyn Woolfolk and Dr. Don Heys for facilitating the structuring of the clinical component of D.D.S./Ph.D. program. Their understanding and willingness to accommodate the demands of the Ph.D. curriculum, while never compromising the quality of the D.D.S. curriculum, allowed me to have an optimal undergraduate dental training experience. I extend thanks to Dr. Don Heys, Dr. Philip Richards, and Dr. Bryan Williams for being influential clinical mentors during my D.D.S. training. I also thank Dr. Peter Yaman, Dr. Gisele Neiva, and Dr. John Heys for acting as clinical mentors throughout my graduate dental training in the Graduate Restorative Dentistry Clinic.

I acknowledge and extend tremendous gratitude to my brothers Ryan and Zachary, who inspired me to apply to the Oral Health Sciences Ph.D. program. Both Ryan and Zach played formative roles in my scientific development and the progression of my doctoral studies. I relied on them to discuss scientific ideas, trouble shoot problems at the bench, and when necessary to travel to Ann Arbor to assist me in performing scientific experiments. I am ever grateful to my parents for providing unconditional love and support. Their commitment to professionalism and helping others was influential in my decision to pursue a career in science and healthcare. The unselfish love, support, and positive encouragement provided by my family members played a critical role in the progression and success of my D.D.S./Ph.D. studies.

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ABSTRACT

PROTEOGLYCAN 4: A DYNAMIC REGULATOR OF PARATHYROID HORMONE BIOLOGIC ACTIONS

By

Chad M. Novince

Chair: Laurie K. McCauley

Intermittent parathyroid hormone (PTH) (1-34) is the only anabolic agent currently approved for the treatment of osteoporosis in the U.S. PTH (1-34) is under clinical investigations as a local bone regenerative therapy and as a potential stem cell therapy following bone marrow transplants. While PTH has known actions increasing bone mass and supporting the expansion of hematopoietic progenitor cells (HPCs), the mechanisms mediating such actions are poorly understood. Most recently it was reported that intermittent PTH protects articular cartilage in degenerating joints.

Proteoglycan 4 (*Prg4*), a novel PTH-responsive gene, was investigated as a potential mediator of PTH biologic actions. *Prg4* protein product actions have been implicated in articular joint protection, HPC expansion, and megakaryopoiesis. Loss-of-function mutations in *PRG4* result in camptodactyly-arthritis-coxa vara-pericarditis syndrome, which is characterized by precocious joint failure and osteopenia. The overall hypothesis that proteoglycan 4 mediates PTH actions in hematopoiesis, skeletal

anabolism, and degenerating joints was investigated using *in vitro* and *in vivo* strategies with *Prg4* null mice.

The investigation of proteoglycan 4 effects on hematopoiesis in adult *Prg4* mutant mice revealed that proteoglycan 4 is an immunomodulatory factor regulating physiologic immune cell populations and PTH actions increasing HPCs. SDF-1 was identified as a strong candidate regulator of proteoglycan 4 actions on hematopoiesis. Skeletogenesis studies demonstrated that proteoglycan 4 supports endochondral bone formation and the attainment of peak trabecular bone mass in the developing skeleton. In the mature remodeling skeleton proteoglycan 4 appears to indirectly support skeletal homeostasis and PTH anabolic actions by protecting joint function. Findings that PTH regulated gene expression similarly in bone and liver highlight the liver as a potential mediator of PTH biologic actions. The joint investigation identified increased joint SDF-1 levels as a novel candidate mechanism mediating articular cartilage degeneration in *Prg4* mutant mice.

This dissertation study of the impact of proteoglycan 4 on the actions of PTH in hematopoiesis, skeletal anabolism, and degenerating joints advances our understanding of the mechanisms mediating PTH biologic actions. Moreover, this body of work further elucidates the role of proteoglycan 4 in physiologic hematopoiesis, skeletogenesis, and joint protection.

CHAPTER 1

INTRODUCTION

Problem Statement

Parathyroid hormone (PTH) plays a prominent role in calcium homeostasis and bone remodeling (1). Intermittent parathyroid hormone (PTH) (1-34) therapy is the only anabolic agent currently approved for the treatment of osteoporosis in the U.S., and is actively under clinical investigation to treat localized osseous defects in the oral cavity and other skeletal sites (2;3). Intermittent PTH (1-34) has been shown to support hematopoietic progenitor cell (HPC) expansion and the mobilization of HPCs (4-6), and as a result is being clinically investigated as a potential stem cell therapy following bone marrow transplants (7). While intermittent PTH administration has proven anabolic actions in bone, its mechanisms are poorly understood. Recent interest has centered on the ability of PTH to stimulate HPCs (4-6), but little is known regarding the mechanisms for this promising activity and whether they are linked to its skeletal actions.

Studies have attributed PTH anabolic actions in the skeleton to mechanisms that extend beyond direct signaling at the PTH/PTH-related protein (PTHrP) receptor (PPR) in osteoblastic cells (8). The production of biologic factors that regulate paracrine signaling between stromal and hematopoietic cells in the bone marrow micro-

environment has been suggested to be integral to its bone forming activity. Additional evidence suggests that hematopoietic cells may support PTH anabolic actions (4;9). While osteoclastic cells have been extensively investigated as potential mediators of PTH anabolism (10-13), more recent studies suggest that HPCs play a key role in regulating PTH anabolic actions (4;9). Of interest, PTH signaling has been shown to regulate the expression of a number of genes which support both HPCs and megakaryocytes (14). The megakaryocyte, an intriguing hematopoietic cell known for platelet production, is a possible candidate regulator of PTH skeletal anabolism.

Proteoglycan 4 (*Prg4*) is a novel PTH responsive gene that was identified in the project laboratory via a gene micro-array study. *Prg4* protein product actions are essential for joint protection, and have been implicated in HPC expansion and the regulation of megakaryopoiesis (15). Loss-of-function mutations in *PRG4* result in the arthropathic and osteopenic condition of campyodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome (16). Of interest, *Prg4* codes for novel protein products which support both HPCs (17) and megakaryopoiesis (18). The role of proteoglycan 4 as a mediator of skeletal development and remodeling, and PTH actions in hematopoiesis and bone is unknown.

Most recently it was reported that intermittent PTH (1-34) protects articular cartilage in degenerating joints (19;20), the mechanisms mediating this novel PTH action are undetermined. In addition to CACP syndrome, PRG4 is reduced in non-inflammatory and inflammatory mediated degenerative joint diseases, including osteoarthritis (OA) (21;22) and rheumatoid arthritis (RA) (23), which suggests that decreased PRG4 levels contribute to degenerative joint disease. The role of proteoglycan 4 as a possible

mediator of putative PTH actions in protecting against articular cartilage degeneration in arthropathic joints is uninvestigated.

General Hypothesis

Three specific aims optimized *in vitro* and *in vivo* strategies built upon the use of *Prg4* null mutant mice to investigate the overall hypothesis that proteoglycan 4 mediates PTH actions in hematopoiesis, skeletal anabolism, and degenerating joints.

Specific Aims

Aim 1: Elucidate the intrinsic role of *Prg4* in osteoblastogenesis.

Hypothesis 1: *Prg4* directly regulates osteoblastogenesis.

Strategies: Primary bone marrow stromal cell cultures, derived from *Prg4* mutant and wildtype mice, were used to investigate the function of *Prg4* in stromal-osteoblastic cell proliferation, differentiation, and cell death. Gene expression studies were performed to evaluate the effects of proteoglycan 4 and PTH on phenotypic osteoblastic and skeletal tissue associated genes.

Aim 2: Determine the role of proteoglycan 4 in the anabolic actions of PTH.

Hypothesis 2: *Proteoglycan 4* is a critical mediator of physiologic bone remodeling and PTH skeletal anabolism, through its regulation of HSCs and megakaryopoiesis.

Strategies: *Prg4* mutant mice were utilized to analyze the effect of proteoglycan 4 in PTH actions in hematopoiesis and skeletal anabolism.

Aim 3: Investigate the impact of proteoglycan 4 in PTH actions in degenerating joints.

Hypothesis 3: *Proteoglycan 4 is required for the chondroprotective actions of PTH in degenerating joints.*

Strategies: *Prg4* mutant mice were used to assess the proteoglycan 4 functions regulating articular cartilage protection, and the impact of PTH on articular cartilage in degenerating joints deficient in proteoglycan 4.

The research is relevant since it will advance the understanding of mechanisms mediating intermittent PTH biologic actions and hence optimize its clinical potential. Moreover, this body of work further elucidates the role of proteoglycan 4 in physiologic hematopoiesis, skeletogenesis, and joint protection.

Background and Significance

General proteoglycan 4 (*Prg4*) background.

The *Prg4* gene encodes for an approximately 345-kD proteoglycan, consisting of 1,404 amino acids spanning 12 exons. *Prg4* mRNA is expressed in skeletal and non-skeletal tissues; including articular joints, bone, liver, heart, and muscle (24). The four isolated *Prg4* protein products; lubricin, superficial zone protein (SZP), hemangiopoietin

(HAPO), and megakaryocyte stimulating factor (MSF) are alternative *Prg4* splice variants, which undergo various post translational modifications (15). proteoglycan 4 is not a classic proteoglycan in the sense that it does not function as a component of the cartilage-matrix (e.g. like aggrecan) or as a transmembrane-cell surface receptor (e.g. like syndecans). Lubricin, SZP, HAPO, and MSF are secreted glycoproteins with diverse biological functions. A proteoglycan 4 receptor has not been identified (17).

Lubricin and SZP are expressed in synovial joints by synoviocytes and superficial zone articular chondrocytes, having lubricating and protective effects (15;25). Characterization of the articular joints in *Prg4* null mice demonstrated that absence of proteoglycan 4 causes hyperplasia of synovial intimal cells, disappearance of superficial zone chondrocytes, abnormal protein deposition on cartilage surfaces, articular cartilage degradation, and ultimately precocious joint failure (15;26). Loss-of-function mutation in *PRG4* result in an autosomal recessive disorder, camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome (16), which is primarily characterized by precocious joint failure. While it has been shown that proteoglycan 4 prevents synoviocyte hyperplasia by restricting adhesion dependent synovial intimal cell proliferation *ex vivo* (15), the mechanism(s) by which proteoglycan 4 protects articular cartilage remain unclear.

While numerous studies have investigated the lubricating and protective functions of lubricin and SZP in the articular joint (15), MSF and HAPO have not been thoroughly investigated. Selected studies have reported that HAPO supports HPC expansion (17;27;28) and MSF regulates megakaryopoiesis (18;29;30), yet the actions of proteoglycan 4 on hematopoiesis are unclear. While studies have demonstrated that *Prg4* is endogenously expressed in bone (24) and loss-of-function mutations in *Prg4* result in

osteopenia (15), there are no known investigations of the actions of proteoglycan 4 in bone. Of interest, *Prg4* is a novel PTH responsive gene in bone and liver (31).

General parathyroid hormone (PTH) background.

Parathyroid hormone (PTH) is an endogenous 84 amino acid hormone produced and secreted by the chief cells of the parathyroid gland in response to decreased extracellular calcium. PTH acts on bone and kidney, via the PTH/PTH-related protein receptor (PPR), to increase extracellular ionized calcium concentration (1). In addition to PTH, PTH-related protein (PTHrP) has the ability to bind PPR, contributing to PTH signaling. PTHrP factors prominently during development, homeostasis, and tumorigenesis (32).

The skeletal biological activity of PTH 1-84 resides in the first 34 amino acids (33) PTH directly signals to osteoblasts by binding the PPR, a seven transmembrane domain G protein coupled receptor. Signaling at the PPR activates the protein kinase A (PKA), protein kinase C (PKC), and mitogen activated protein kinase (MAPK) signal transduction pathways (34). PKA, PKC, and MAPK activate immediate early response genes, which form transcription factor complexes that regulate the expression of downstream osteoblastic genes. More recently PTH signaling via PKA and PKC has been shown to intersect with the Wnt pathway in osteoblasts, supporting canonical Wnt signaling (35;36).

Exogenous PTH administration has both catabolic and anabolic actions on bone, depending on the method of administration. Continuous PTH administration induces bone resorption, while intermittent PTH administration stimulates bone formation. Teriparatide (PTH 1-34) was approved by the FDA in 2002 for the treatment of

osteoporosis, under the brand name, Forteo™ and is currently the only FDA approved anabolic osteoporosis treatment in the U.S. Teriparatide is also under investigation for the treatment of localized osseous defects, such as periodontal defects and fracture healing (2;3). Findings that PTH supports hematopoietic progenitor cell (HPC) expansion and the mobilization of HPCs have led to the investigation of PTH as a potential stem cell therapy following bone marrow transplants (4-6). Most recently, rodent OA studies have demonstrated that PTH (1-34) inhibits articular cartilage degeneration in arthropathic joints.

Proposed mechanisms for the anabolic actions of PTH: Osteoblastogenesis.

Intermittent PTH exerts its anabolic effects via increasing the number of osteoblasts, through mechanisms which include stimulating preosteoblast proliferation, promoting preosteoblast and osteoblast differentiation and inhibiting osteoblast apoptosis (33;37-39). However, whether the proliferation of preosteoblasts is increased with PTH administration remains controversial (40-43). The anti-apoptotic effects of PTH on osteoblasts are dependent on the stage of differentiation of osteoblastic cells. In preconfluent cells, PTH protected against induced reduction in cell viability through the activation of cAMP, whereas in postconfluent cells pro-apoptotic effects were observed (44). Interestingly, *in vitro*, the direct effect of PTH or PTHrP on osteoblastic cells is to inhibit osteoblastic phenotypic markers and restrict differentiation. A key component of these actions is that the direct effect of PTH on osteoblastic cells is to inhibit their mineralization and bone formation capacity (8). This suggests that an intermediary cell is critical in PTH anabolic actions in bone.

Other proposed PTH mechanisms (mechanical, growth factors, transcriptional mediators).

PTH anabolism and mechanical stimulation are interrelated. PTH potentiates mechanically induced expression of second messenger signaling molecules, including 1,4,5-inositol triphosphate (IP3), PKC, cAMP (45), and intracellular calcium (Ca²⁺) *in vitro* (46). *In vivo* mechanical loading studies have shown that intermittent PTH administration enhances mechanical stimulation of new bone formation (47-51). Skeletal unloading intermittent PTH administration studies result in an attenuation of PTH anabolic actions (52-56). PTH signaling in osteoblastic cells has been shown to regulate the expression of several growth factors. Most notably, PTH anabolic actions have been linked to insulin-like growth factor I (IGF-I) and basic fibroblast growth factor 2 (FGF-2). Single PTH administration to murine cultured osteoblastic cells rapidly up-regulates FGF-2 mRNA (57), and *in vivo* intermittent PTH administration increases IGF-I mRNA (58). IGF-I deficient and IGF-I receptor deficient mice do not respond to intermittent PTH (59-62), and FGF-2 deficient mice have a significantly impaired anabolic PTH response (63). Transcriptional mediators such as c-Fos have also been implicated in PTH anabolic actions, which may be associated with its role in cells of the osteoclast lineage (64). Runx2 has been implicated as a transcriptional mediator of PTH anabolic effects via its actions on sclerostin (65;66). Nmp4/CIZ (nuclear matrix protein 4/cas interacting zinc finger protein), a ubiquitously expressed nucleocytoplasmic shuttling transcription factor, has recently been implicated as a transcriptional suppressor of PTH anabolism (67).

PTH and hematopoietic cells.

There has been a great deal of interest in the impact of bone cells, in particular how osteoclasts differentiate from hematopoietic cells and affect bone homeostasis. PTH and/or PTHrP signaling in osteoblastic cells regulate the expression of osteoclastic cytokines, most notably receptor activator of nuclear factor- κ B ligand (RANKL) and osteoprotegerin (OPG). A putative role for cells of the osteoclast lineage as regulators of PTH anabolism has been suggested but is still quite controversial with lines of evidence pro and con (10-13). The concept of cells of the osteoclast lineage associated with a specific temporal phase of differentiation is also an emerging theme (11;13;68).

More recently, a growing area of interest is how osteoblasts impact hematopoiesis (69). A relatively unexplored area is how hematopoietic cells in the marrow impact bone formation. Cytokine production by hematopoietic cells impacts osteoblastic activity but whether via direct cell contact or juxtacrine factors is not clearly defined. Recent interest has focused on the potential role of PTH and PTHrP as a stem cell factor that supports hematopoiesis. Work with a constitutively active PPR revealed that PTH signaling resulted in HSC expansion via Jagged1 ligand, and increased trabecular bone formation while cortical bone was reduced (70). Further investigation revealed that intermittent PTH supported hematopoiesis through its action on early HPCs. More recently, *ex vivo* and *in vivo* PTH studies using the global IL-6 knock-out mouse demonstrated that IL-6 supports PTH expansion of HPCs (71). Furthermore, it was reported that PPR expressing T-lymphocyte cells amplify PTH anabolic actions through Wnt10b signaling (9). The role of accessory hematopoietic cells in bone is emerging as a strong candidate for anabolic actions of PTH.

Megakaryocytes are hematopoietic cells with potential for mediating PTH anabolic actions. PTH and/or PTHrP regulate the expression of a number of hematopoietic genes which dually support HPCs and megakaryocytes. Such genes include interleukin-6 (IL-6) (72-75), stem cell factor (SCF) (26;76), fibroblast growth factor-2 (FGF-2) (77), and stromal derived factor-1 (SDF-1) (78;79). Recent studies of megakaryocyte development have demonstrated their prominent role in HSC biology. While the connection between megakaryocytes and HPCs remains unclear, the cells have common surface receptors (c-mpl, CXCR4, CD150, CD41, etc.) lineage specific transcription factors (Runx-1, GATA-2, Evi-1, SCL/TAL1) and specialized signaling pathways (Notch signaling, prostaglandins) (14).

Megakaryocytes are polyploid cells present in the bone marrow, defined by their biologic role in thrombopoiesis. Megakaryocytes have been found to support osteoblastogenesis (80-82) and inhibit osteoclastogenesis (62;64) and mice with aberrations in their megakaryocytes have prominent increased bone phenotypes. NF-E2 and GATA-1 null mutant mice, deficient in transcriptional mediators of megakaryopoiesis, have large numbers of immature megakaryocytes and dysfunctional platelets leading to thrombocytopenia (83;84). Interestingly, these mice have an increased bone phenotype that is observed only in bony sites of hematopoiesis. To our knowledge prior studies have not examined PTH actions in megakaryopoiesis, or the role of the megakaryocyte as a regulator of PTH anabolic actions.

In summary, PTH clearly targets not only the osteoblastic cells but also the hematopoietic cells that impact the microenvironment through their potential to mediate cell communication, commitment and differentiation. This area of research is rapidly

changing and although there are many 'candidate' mediators for PTH action identified using rationale model systems, there is no convergence regarding the mechanistic trajectory of PTH and the critical mediators of its action.

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CHAPTER 2

PROTEOGLYCAN 4, A NOVEL IMMUNOMODULATORY FACTOR, REGULATES PARATHYROID HORMONE ACTIONS ON HEMATOPOIETIC CELLS

Abstract

Proteoglycan 4 (*Prg4*), a critical protective factor in articular joints, has been implicated in hematopoietic progenitor cell expansion and megakaryopoiesis. *PRG4* loss-of-function mutations result in campptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, which is primarily characterized by precocious joint failure. *Prg4* was identified as a novel parathyroid hormone (PTH) responsive gene in bone, and was investigated as a potential mediator of PTH actions on hematopoiesis. *Prg4* mutant and wildtype mice were treated daily with intermittent PTH (1-34) or vehicle control for 6 weeks, from 16-22 weeks of age. Twenty-two week old *Prg4* mutant mice had increased peripheral blood neutrophils and decreased marrow B220⁺ (B-lymphocytic) cells, which were normalized by PTH. The PTH-induced increase in marrow Lin⁻Sca-1⁺c-Kit⁺ (hematopoietic progenitor) cells was blunted in mutant mice. Basal and PTH-stimulated stromal cell-derived factor-1 (SDF-1) was decreased in mutant mice suggesting it as a candidate regulator of proteoglycan 4 actions on hematopoiesis *in vivo*. PTH-stimulated IL-6 mRNA was of greater magnitude in mutant versus wildtype calvaria and bone marrow suggesting a compensatory mechanism in the PTH-induced increase in marrow

hematopoietic progenitor cells. In summary, *Prg4* is a novel PTH responsive factor regulating immune cells and PTH actions on marrow hematopoietic progenitor cells.

Introduction

Parathyroid hormone (PTH), best known for its roles in calcium homeostasis and bone remodeling (1), has been shown to support hematopoietic progenitor cell (HPC) expansion and the mobilization of HPCs (2-4). While clinical investigations are assessing intermittent PTH administration as a potential stem cell therapy following bone marrow transplants (5), the mechanisms mediating PTH actions on hematopoiesis are poorly understood.

Stromal/osteoblastic cells are the dominant PTH/PTH-related protein (PTHrP) receptor (PPR) expressing cells in the bone marrow. With the exception of specific PPR expressing T-cell sub-populations (6;7), PTH actions on hematopoiesis are believed to be mediated via paracrine or juxtacrine signaling from PPR activated stromal/osteoblastic cells (1;2;8).

Extensive studies have demonstrated that PPR signaling in stromal/osteoblastic cells alters the expression of osteoclastic cytokines, most notably receptor activator of nuclear factor- κ B ligand (RANKL) and osteoprotegerin (OPG), which regulate the differentiation of hematopoietic cells to mature bone resorbing osteoclastic cells (8-12). More recent studies have focused on the role of PTH actions on HPCs. Calvi et al. (2003) demonstrated in mutant mice with osteoblast-specific constitutively active PPR that osteoblastic cells increase HPC expansion via Jagged1 ligand (2). Further

investigation revealed that intermittent PTH administration in normal mice supported hematopoiesis through actions on early HPCs (2). *Ex vivo* and *in vivo* PTH studies using the global IL-6 knock-out mouse demonstrated that IL-6 supports PTH expansion of HPCs (13).

Proteoglycan 4 (*Prg4*), a gene that has been implicated in HPC expansion (14-16) and megakaryopoiesis (17-19), was identified as a novel PTH responsive gene in bone. The *Prg4* gene encodes an approximately 345-kD proteoglycan, consisting of 1,404 amino acids spanning 12 exons. *Prg4* is expressed across skeletal and non-skeletal tissues, with highest relative levels of expression in articular joints, liver, and bone (20;21). The four isolated *Prg4* protein products, lubricin, superficial zone protein (SZP), hemangiopoietin (HAPO), and megakaryocyte stimulating factor (MSF), are secreted glycoproteins which have been implicated in articular joint protection (21;22), HPC expansion (14), and megakaryopoiesis (17) (**Table 2.1**). A proteoglycan 4 receptor has not yet been identified (14;21). Loss-of-function mutations in *PRG4* result in the human autosomal recessive disorder, camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, which is characterized by early onset joint failure and osteopenia. CACP patients have not been reported to spontaneously develop hematological conditions, nor have there been any reported immune cell abnormalities (23).

While selected studies have reported that HAPO supports HPC expansion (14-16) and MSF regulates megakaryopoiesis (17-19), the actions of proteoglycan 4 on hematopoiesis are unclear. The purpose of this study was to investigate the unexplored role of proteoglycan 4 as a regulator of physiologic hematopoiesis and PTH actions on

hematopoiesis in the mature skeleton. The use of the *Prg4* null mouse model (21) provides for the first known study of *Prg4* actions on hematopoiesis *in vivo*.

Materials and Methods

C57BL6 wildtype mice: Single PTH administration

Sixteen week old C57BL6 wildtype mice were administered a single subcutaneous injection of recombinant human PTH (1-34) (1 $\mu\text{g/g}$) (Bachem, Torrence, CA) or vehicle (0.9% NaCl), sacrificed 4, 8, or 12 hours later, and bone marrow was harvested for gene expression analysis. All animal studies were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA), and animals were maintained in accordance with approved UCUCA research protocols.

Primary calvarial osteoblastic cell cultures: Single PTH administration

Primary osteoblasts were isolated from calvaria harvested from 7-14 day old C57BL6 wildtype mice. Briefly, calvaria were dissected, isolated from periosteum, and subjected to sequential digestions of 10, 20 and 60 minutes in α -modified minimum essential medium (α -MEM) (Invitrogen, Carlsbad, CO) containing collagenase A (2 mg/ml) (Roche Molecular Biochemicals, Indianapolis, IN) and 0.25% trypsin (Invitrogen). Cells from the last digest were washed, counted, and plated at $5 \times 10^4/\text{cm}^2$ in α -MEM with 10% fetal bovine serum (FBS) (HyClone, Provo, UT), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. Cells were induced to differentiate via supplementing medium with 50 $\mu\text{g/ml}$ ascorbic acid and 10mM β -glycerophosphate.

Cultures (day 5) were administered a single recombinant human PTH (1-34) (10 nM) (Bachem, Torrance, CA) or vehicle (4mM HCl/0.1%BSA) treatment, and harvested 8, 12, or 24 hours later for gene expression analysis. In another experimental protocol, d1, d5, d7, and d14 cultures were administered a single recombinant human PTH (1-34) (10 nM) (Bachem, Torrance, CA) or vehicle (4mM HCl/0.1%BSA) treatment, and harvested 4 hours later for gene expression analysis.

***Prg4* mutant (-/-) mice: Background**

Prg4 mutant (-/-) mice, generated via the creation of a mutant *Prg4* allele by homologous recombination in 129Sv/Ev-derived embryonic stem cells, were generously provided by Matthew Warman (Harvard) (21). Mutant mice were backcrossed from the 129Sv/Ev genetic background to the C57BL6 genetic background. A PCR-based assay was used to genotype the mice as previously described (21).

***Prg4* mutant (-/-) mice: Single PTH administration**

Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) littermate mice were administered a single subcutaneous injection of recombinant human PTH (1-34) (1 µg/g) (Bachem) or vehicle (0.9% NaCl), sacrificed 0 (no treatment control), 1, 4, 8, or 12 hours later, and bone marrow and calvaria were harvested for gene expression analysis.

***Prg4* mutant (-/-) mice: Six weeks daily intermittent PTH administration**

In an intermittent PTH experimental protocol, sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) littermate mice were administered intermittent daily subcutaneous injection of recombinant human PTH (1-34) (50 µg/kg) (Bachem) or vehicle (0.9% NaCl) control for 6 weeks, from 16-22 weeks. Twenty-four hours following the final injection, twenty-two week old mice were sacrificed and tissues harvested for analyses.

Quantitative real-time PCR

Bone marrow was directly flushed from femur and tibia with TRIzol reagent (Invitrogen) with a 3 ml syringe and 22G ½ inch needle. Calvaria were flash frozen, pulverized, and homogenized in TRIzol reagent. Calvarial osteoblast cultures were washed three times with 1X PBS, and TRIzol was directly applied to cultures. In each case, RNA was isolated following manufacturer's directions, and total RNA was quantified. Double-stranded cDNA was synthesized from 1.0 µg of RNA, using Random Hexamers (Applied Biosystems, Branchburg, NJ) and Multiscribe Reverse Transcriptase (Applied Biosystems). cDNA was amplified using the TaqMan Universal PCR Master Mix (Applied Biosystems) with TaqMan gene expression specific primers-probes (Applied Biosystems) for proteoglycan-4 (*Prg4*), interleukin-6 (IL-6), thrombopoietin (TPO), stromal cell-derived factor-1 (SDF-1), and osteocalcin (OCN). Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) was used as an endogenous control. Amplification was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative quantification of data was carried out using the standard curve method or the comparative CT method (24).

Complete blood counts

Whole blood was collected by cardiac puncture, transferred to Microtainer tubes with potassium-EDTA (BD Biosciences, San Jose, CA) and gently agitated. Complete blood count analysis (automated differential) was performed using the Hemavet 1500FS (Drew Scientific, Waterbury, CT).

Flow cytometry

Flushed femoral bone marrow cells and dissociated spleen cells were isolated, and all staining steps were performed using flow cytometry staining buffer (FSB) (1X PBS, 2% FBS, 2mM EDTA). Femurs were sectioned at the mid-diaphysis, a 22G ½ inch needle was gently rotated through the epiphysis into the marrow cavity, and marrow cells were flushed out the sectioned mid-diaphysis with FSB. Spleens were incised lengthwise, placed on a cell strainer and disassociated with a 1 mL syringe plunger while simultaneously delivering FSB. Cell suspensions were homogenized, strained, centrifuged, re-suspended and cell counts performed. Three million cells were suspended in 0.5 mL FSB, incubated with fluorochrome conjugated antibodies (BD Biosciences) for 30 minutes at 4°C, washed, centrifuged, and re-suspended in 1 mL FSB containing 7-AAD (Invitrogen) for analysis. All antibodies were acquired from BD Biosciences. The frequency of CD11b⁺GR1⁺ (immature myeloid) cells, CD3⁺ (T-lymphocytic) cells, B220⁺ (B-lymphocytic) cells, Lin⁻Sca-1⁺c-Kit⁺ (hematopoietic progenitor) cells, and CD41⁺ (megakaryopoietic) cells were assessed in bone marrow. The frequency of B220⁺ (B-lymphocytic) cells was assessed in spleen. Samples were analyzed using a FACSCalibur Flow Cytometer and CellQuest Pro Software (BD Biosciences).

A flow cytometry double-staining technique was used to assess megakaryocyte ploidy. Three million femoral bone marrow cells were suspended in 0.5 mL FSB, incubated with FITC-conjugated CD41 antibody for 30 minutes at 4°C, rinsed with 1X PBS, centrifuged, and fixed in 1 mL 1% paraformaldehyde overnight at 4°C. Cells were centrifuged, washed with 1X PBS, re-suspended in 1 mL propidium iodide (PI) staining solution (1X PBS, 4mM sodium citrate, 0.1% Triton X-100, 50 µg/mL PI, and 100

µg/mL RNase A), and incubated for 1 hour at 37°C. At least 10,000 gated cells were analyzed for each sample. Samples were analyzed using a FACSCalibur Flow Cytometer (BD Biosciences) and CellQuest Pro Software (BD Biosciences).

Histomorphometry

Tibiae were fixed in 10% phosphate-buffered formalin for 48 hours at 4°C, dehydrated in graded ethanols and xylene, and embedded undecalcified in modified methylmethacrylate. Frontal serial sections (4 µm) were cut with vertical bed microtomes (Leica/Jung 2065 and 2165 Bannockburn, IL) and affixed to slides pre-coated with 1% gelatin solution. Sections were stained according to the von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA), and used to enumerate megakaryocytes per marrow area. Histomorphometric data were collected semi-automatically with a light Nikon microscope and the OsteoMeasure/Trabecular Analysis System (OsteoMetrics Inc., Atlanta, GA).

Tibiae, liver (left lobe), and spleen were fixed in 10% phosphate-buffered formalin for 48 hours at 4°C. Tibiae were decalcified in 14% EDTA for 14 days at room temperature. Tibiae, liver, and spleen were embedded in paraffin, and 5 µm serial sections were cut and stained. Hematoxylin & eosin (H&E) stain was performed in all tissue sections.

Stromal cell-derived factor-1 (SDF-1) immunofluorescence (IF) stain was performed in proximal tibia sections. Samples were deparaffinized with mixed xylenes, re-hydrated in graded ethanols, then briefly washed with PBT (PBS plus 0.2% Triton X-100) and blocked for 30 minutes with Image-iT FX Signal Enhancer (Invitrogen). SDF-1 IF was performed via Zenon Alexa Fluor 488 Rabbit Labeling Kit (Invitrogen) and SDF-

1 alpha rabbit polyclonal antibody (Catalogue number: ab25117) (ABCAM, Cambridge, MA). After overnight incubation at 4° with 1 ug/100ul dilution of fluorescence- labeled SDF-1 alpha rabbit polyclonal antibody (ABCAM), sections were washed with PBT, mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), and cover slipped. Images were acquired with Olympus FV-500 confocal microscope. Image analysis of SDF-1 IF in proximal tibia trabecular bone (secondary spongiosa) was performed using Image Pro Plus 5.1 Software (Media Cybernetics, Silver Spring, MD).

Bone marrow biochemical studies

Bone marrow was directly flushed from femur and tibia with 1 mL 0.9% NaCl using a 1 mL syringe and 22G ½ inch needle. The marrow suspension was homogenized and cell counts performed. Cell suspensions were centrifuged at 1000 RPM for 5 minutes, supernatants were collected, stored at -80°C, and freeze-thawed once. Stromal cell-derived factor-1 (SDF-1) levels in the bone marrow supernatant were analyzed by antibody sandwich ELISA (R&D Systems, Minneapolis, MN) per manufacturer's protocol with a detection range of 62.5 to 10000 pg/ml. SDF-1 levels were normalized by marrow cell numbers.

Statistical analysis

Analysis of variance (ANOVA) or unpaired *t* test were performed using GraphPad InStat Software (GraphPad Software, San Diego, CA). Statistical significance was noted at $p < 0.05$ or lower. Data are presented as mean \pm standard error of mean (SEM).

Results

Single PTH administration increased *Prg4* mRNA levels in bone

Quantitative real-time PCR studies demonstrated that *Prg4* is a novel PTH responsive gene in bone, *in vivo* and *in vitro* (**Figure 2.1A-C**). A single subcutaneous injection of PTH (1-34) (1 μ g/g) in sixteen week old C57BL6 wildtype mice significantly increased *Prg4* mRNA in bone marrow at 4 and 8 hours after injection (**Figure 2.1A**). Moreover, a single PTH (1-34) administration (10 nM) to primary calvarial osteoblastic cell cultures significantly up-regulated *Prg4* mRNA at 8, 12 and 24 hours following treatment (**Figure 2.1B**).

Prg4 mRNA expression was evaluated in d1, 5, 7, and 14 primary calvarial osteoblastic cell cultures to elucidate the role of osteoblast maturation on basal and PTH-stimulated *Prg4* mRNA expression. Basal *Prg4* mRNA expression was highest in less differentiated osteoblastic cell cultures and significantly decreased with increasing differentiation over time from d1 to d5 ($p < 0.001$), d5 to d7 ($p < 0.01$), and d7 to d14 ($p < 0.05$) (**Figure 2.1C**). A single 4 hour PTH administration significantly increased *Prg4* mRNA in d1, d5, d7, and d14 cultures. Relative to vehicle-control samples, PTH increased *Prg4* mRNA more significantly in d14 (10.20-fold) and d7 cultures (10.66-fold) vs. d5 (2.48-fold) and d1 (1.43-fold) cultures ($p < 0.001$ and $p < 0.001$ respectively) (**Figure 2.1C**). Osteocalcin gene expression studies validated the maturation of osteoblastic cells in culture over time as we have previously published (25) (**data not shown**).

Bone marrow, liver, and spleen morphology

Tibia, liver, and spleen were isolated after 6 weeks of PTH or vehicle treatment (i.e. twenty-two week old mice) for histological evaluation of hematopoietic organs (**Figure 2.2A-C**). Proximal tibia marrow morphology was similar in twenty-two week old *Prg4* *-/-* vs. *+/+* mice (**Figure 2.2A**). Likewise, there were no morphologic differences found in twenty-two week old *Prg4* *-/-* liver (**Figure 2.2B**) or spleen (**Figure 2.2C**) relative to *Prg4* *+/+* littermates, and no evidence of extramedullary hematopoiesis in these organs.

Bone and marrow IL-6, thrombopoietin and SDF-1 mRNA in *Prg4* *-/-* mice

Based upon prior publications, which have implicated the *Prg4* protein products HAPO and MSF in HPC expansion (14-16) and megakaryopoiesis (17-19), the expression of several genes that are dual regulators of HPCs and megakaryopoiesis were evaluated. Sixteen week old *Prg4* *-/-* and *+/+* mice were administered a single subcutaneous injection of PTH (1-34) (1 μ g/g) or vehicle (0.9% NaCl) control, sacrificed 1, 4, 8, or 12 hours later, bone marrow total RNA was isolated, and quantitative real-time PCR was performed to assess interleukin-6 (IL-6), thrombopoietin (TPO), and stromal cell-derived factor-1 (SDF-1) mRNA expression.

IL-6 is a pleiotropic factor that has diverse biologic roles, which include the support of hematopoiesis, lymphopoiesis (26;27), and megakaryopoiesis (28;29). Marrow IL-6 mRNA levels were significantly lower in control sixteen week old *Prg4* *-/-* mice, and consistently expressed at 60% of the levels detected in *+/+* littermates (**Figure 2.3A**). IL-6 is an immediate early response gene up-regulated in bone *in vitro* and *in vivo* by PTH thirty minutes to one hour after administration (30-32). IL-6 was significantly

increased one hour after PTH injection in sixteen week old *Prg4* *-/-* and *+/+* marrow and was returning to baseline at four hours. Relative to vehicle-control samples, PTH injection increased IL-6 mRNA to a significantly greater extent at one hour in *Prg4* *-/-* marrow (13-fold) vs. *+/+* marrow (5-fold) ($p < 0.01$) (**Figure 2.3A**).

IL-6 gene expression studies were performed in calvaria harvested from untreated and single PTH-treated sixteen week old *Prg4* *-/-* and *+/+* mice to better identify the primary cell population mediating the exacerbated PTH-induced increase in marrow IL-6 mRNA in *Prg4* *-/-* mice. Relative to untreated controls, PTH injection induced a significantly greater increase in calvaria IL-6 mRNA in *Prg4* *-/-* (132-fold) vs. *+/+* mice (45-fold) one hour after treatment ($p < 0.05$) (**Figure 2.3B**).

TPO is the primary factor regulating megakaryopoiesis, and has been shown to support HPCs (26). Marrow TPO mRNA expression was similar in control sixteen week old *Prg4* *-/-* and *+/+* mice, and PTH did not alter marrow TPO mRNA levels in *Prg4* *-/-* or *+/+* mice (**Figure 2.3C**).

SDF-1, a chemokine expressed at high levels by bone marrow stromal and osteoblastic cells, regulates the homing and mobilization of marrow HPCs (34;35), megakaryocyte progenitor cells (27), and immune cell populations (28;29). While intermittent PTH treatment has been reported to increase SDF-1 expression in bone, *in vitro* and *in vivo* (30), little is known regarding the temporal effects of PTH on SDF-1 mRNA expression. Four hours after PTH injection, marrow SDF-1 mRNA levels were similarly reduced in sixteen week old *Prg4* *-/-* and *+/+* mice. Twelve hours following PTH injection, marrow SDF-1 mRNA was significantly increased in *Prg4* *+/+* mice (**Figure 2.3D**), but notably, PTH failed to increase marrow SDF-1 mRNA in *Prg4* *-/-*

mice. Gene expression studies in calvaria, harvested from untreated and single PTH-treated sixteen week old *Prg4* ^{-/-} and ^{+/+} mice, demonstrated no change in SDF-1 mRNA expression at twelve hours after treatment (**data not shown**).

***Prg4* ^{-/-} mice have altered immune cell populations, normalized by PTH**

Whole blood was collected from twenty-two week old PTH and vehicle-treated *Prg4* ^{-/-} and ^{+/+} mice, and complete blood count studies were performed to assess peripheral blood cell populations. There were no differences in red blood cell values or platelet numbers in *Prg4* ^{-/-} vs. ^{+/+} mice (**Table 2.2**). *Prg4* ^{-/-} mice had altered peripheral white blood cells, which included increased percentages of neutrophils and decreased percentages of lymphocytes (**Figure 2.4A,B**) (**Table 2.2**). Unexpectedly, PTH treatment normalized the frequency of peripheral blood neutrophils and lymphocytes in *Prg4* ^{-/-} mice to ^{+/+} levels (**Figure 2.4A,B**) (**Table 2.2**). Analysis of absolute cell numbers revealed that *Prg4* ^{-/-} mice had increased numbers of neutrophils that were normalized by PTH (**Figure 2.4C,D**) (**Table 2.2**).

To elucidate whether differences in peripheral blood immune cells reflected changes in the marrow, femoral bone marrow was harvested from twenty-two week old *Prg4* ^{-/-} and ^{+/+} mice for flow cytometric analysis of CD11b⁺GR1⁺ (immature myeloid) cells, CD3⁺ (T-lymphocytic) cells, and B220⁺ (B-lymphocytic) cells. The frequency of marrow CD11b⁺GR1⁺ cells (**Figure 2.5A**) and CD3⁺ (T-lymphocytic) cells (**Figure 2.5B**) did not differ in *Prg4* ^{-/-} vs. ^{+/+} mice. Similar to the peripheral blood lymphocyte findings, *Prg4* ^{-/-} mice had a reduced percentage of marrow B220⁺ (B-lymphocytic) cells, which were normalized to ^{+/+} levels with PTH treatment (**Figure 2.5C,D**). Based upon differences in peripheral blood lymphocytes and marrow B-lymphocytic cells, the

frequency of B220⁺ (B-lymphocytic) cells was assessed in the spleen, a center for B-lymphopoiesis. The frequency of B220⁺ (B-lymphocytic) cells in spleen was similar in twenty-two week old *Prg4*^{-/-} and *+/+* mice (**Figure 2.5C,E**). PTH decreased the percentage of splenic B220⁺ cells in *Prg4*^{+/+} mice, an effect that may be secondary to PTH actions supporting the expansion of HPCs in the spleen (31). These data suggest that proteoglycan 4 restricts peripheral blood neutrophil numbers, which does not appear to be secondary to an altered frequency of marrow immature myeloid cells. Moreover, these data indicate that proteoglycan 4 supports the frequency of marrow B-lymphocytic cells.

***Prg4*^{-/-} mice had decreased expansion of marrow HPCs induced by PTH**

Prior studies reported that recombinant HAPO administration increased marrow HPCs in normal mice (14) and accelerated marrow hematopoietic reconstitution in mice subjected to irradiation (14) or high-dose chemotherapy (15). The frequency of marrow Lin⁻Sca-1⁺c-Kit⁺ (hematopoietic progenitor) cells in twenty-two week old *Prg4*^{-/-} and *+/+* mice was assessed by flow cytometric analysis to investigate the impact of proteoglycan 4 on marrow HPCs (**Figure 2.6A,B**). The percentage of marrow Lin⁻Sca-1⁺c-Kit⁺ cells was similar in vehicle-treated *Prg4*^{-/-} vs. *+/+* mice. PTH increased the frequency of Lin⁻Sca-1⁺c-Kit⁺ cells in both *Prg4*^{-/-} and *+/+* marrow, however this increase was blunted in *Prg4*^{-/-} mice relative to *+/+* littermates (**Figure 2.6A,B**). These data suggest that proteoglycan 4 supports PTH actions increasing marrow HPCs.

Marrow megakaryopoiesis was similar in *Prg4*^{-/-} and *+/+* mice

While there are no reported MSF studies *in vivo*, administration of purified MSF *in vitro* has been suggested to support the formation of platelet forming cells (17-19). To

elucidate the impact of proteoglycan 4 on megakaryopoiesis, the frequency of marrow CD41+ (megakaryopoietic) cells and megakaryocyte ploidy (a relative measure of megakaryocyte maturity) were evaluated in twenty-two week old *Prg4* *-/-* vs. *+/+* mice via flow cytometric analysis. The frequency of marrow CD41+ (megakaryopoietic) cells (**Figure 2.7A**) and megakaryocyte ploidy (**Figure 2.7B**) were similar in *Prg4* *-/-* vs. *+/+* mice. Numbers of megakaryocytes per marrow area in proximal tibia sections were similar in twenty-two week old *Prg4* *-/-* vs. *+/+* mice (**Figure 2.7C,D**). These findings indicate that proteoglycan 4 is not a critical regulator of marrow megakaryopoiesis *in vivo*.

***Prg4* *-/-* mice have altered marrow IL-6 mRNA and SDF-1**

Marrow IL-6 mRNA expression was measured in twenty-two week old *Prg4* *-/-* and *+/+* mice (**Figure 2.8A**) since control sixteen week old *Prg4* *-/-* mice had decreased IL-6 levels relative to *+/+* littermates. Similar to the sixteen week old *Prg4* *-/-* mice, control twenty-two week old *Prg4* *-/-* mice had decreased marrow IL-6 mRNA levels as compared to *+/+* littermates (**Figure 2.8A**). These data suggest that *Prg4* signaling supports basal IL-6 mRNA expression in the bone marrow.

Based upon the findings that twenty-two week old *Prg4* *-/-* mice have increased numbers of peripheral neutrophils, decreased frequency of marrow B-lymphocytes, and a blunted PTH-induced increase in marrow HPC frequency we assessed marrow SDF-1 mRNA and protein expression in twenty-two week old *Prg4* *-/-* vs. *+/+* mice.

Quantitative real-time PCR analysis demonstrated similar marrow SDF-1 mRNA levels in *Prg4* *-/-* and *+/+* mice (**Figure 2.8B**), which indicates that *Prg4* does not impact basal SDF-1 mRNA expression in the marrow. SDF-1 immunofluorescence (IF) analysis in

proximal tibia (**Figure 2.8C,D**) and SDF-1 ELISA analysis of hindlimb marrow (**Figure 2.8E**) demonstrated that SDF-1 protein was reduced in the marrow of *Prg4* *-/-* vs. *+/+* mice. PTH significantly increased SDF-1 protein expression in the marrow of *Prg4* *-/-* and *+/+* mice; however, this was blunted in *Prg4* *-/-* mice relative to *+/+* littermates (**Figure 2.8C-E**). These data indicate that proteoglycan 4 supports basal marrow SDF-1 protein expression and the ability of PTH to increase SDF-1 protein expression in the bone marrow.

Discussion

Prg4 is expressed in the bone marrow of the mature skeleton, which suggests that proteoglycan 4 may impact adult hematopoiesis. That basal *Prg4* mRNA expression was higher in less differentiated osteoblastic cell cultures suggests that more immature osteoblastic cells are a primary source of proteoglycan 4 in the bone marrow. Taking into consideration that more immature osteoblastic cells better promote HPC expansion and function (32), the finding that basal *Prg4* mRNA expression is higher in less differentiated osteoblastic cell cultures suggests that increased proteoglycan 4 expression may be one mechanism by which more immature osteoblastic cells support HPCs.

The *in vivo* PTH-induced increase in bone marrow *Prg4* mRNA indicates that proteoglycan 4 could be a mediator of PTH biologic actions in marrow hematopoiesis. Based upon the PTH-induced increase in *Prg4* mRNA in isolated osteoblastic cell cultures, we speculate that direct PTH signaling at PPR expressing osteoblastic cells increases proteoglycan 4 secretion in the marrow, mediating paracrine signaling to

hematopoietic cells. The finding that PTH increased *Prg4* mRNA more significantly in further differentiated osteoblastic cell cultures may simply reflect the PPR activity that is greater in more differentiated osteoblastic cells (25).

Dysregulated immune cell populations in *Prg4* mutant mice may be secondary to the decreased basal expression of marrow SDF-1 and marrow IL-6 mRNA. Marrow and peripheral neutrophils express the SDF-1 receptor CXCR4 and have been shown to biologically respond to SDF-1 (28;33-35). Studies have demonstrated that the CXCR4/SDF-1 chemokine axis regulates circulating neutrophil homeostasis (37;38;45). Injection of CXCR4 blocking antibody in mice mobilized neutrophils from the marrow (28), and drugs targeting CXCR4 induced a peripheral neutrophilia in mice and humans (36). Since the *Prg4* mutant mouse does not display signs of acute inflammation, which would induce the mobilization of immature neutrophils from the marrow into circulation, we speculate that decreased marrow SDF-1 disrupts the neutrophil CXCR4/SDF-1 axis resulting in increased circulating neutrophils in the *Prg4* mutant mouse.

The decreased frequency of peripheral blood lymphocytes in *Prg4* mutant mice appears to be secondary to increased absolute numbers of peripheral blood neutrophils. Nevertheless, the frequency of marrow B-lymphocytic cells was significantly decreased in *Prg4* mutant mice. While the slight decrease in *Prg4* mutant marrow B220+ cells implies that proteoglycan 4 is not a critical regulator of marrow B-lymphocyte cell numbers under physiologic conditions, proteoglycan 4 actions supporting B220+ cell numbers may play a more crucial role during immune response.

The decreased expression of marrow SDF-1 in *Prg4* mutant mice may contribute to the reduced frequency of marrow B-lymphocytic cells. B-lymphocyte progenitor cells

express the CXCR4 receptor at high levels (37;38). Mice deficient in SDF-1 have severely reduced B-lymphocyte progenitors in the marrow (39;40), and studies have demonstrated that SDF-1 supports the homing (38;46;47) and proliferation (41) of marrow B-lymphocyte progenitors. Furthermore, reduced marrow IL-6 mRNA expression in *Prg4* mutant mice may contribute to decreased frequency of B-lymphocytic cells since IL-6 supports physiologic B-lymphopoiesis (42-44). Similar to *Prg4* mutant mice, mice with an osteoprogenitor cell $G_{s\alpha}$ deficiency (a downstream mediator of the PPR) have decreased peripheral blood and marrow B-lymphocytic cells (45), suggesting osteoblast cell signaling could also play a role in *Prg4* mutant mice. The unanticipated finding that PTH induced normalization of peripheral blood neutrophils and marrow B-lymphocytic cells in *Prg4* mutant mice to wildtype levels may be associated with the PTH induced increase in marrow SDF-1 protein in *Prg4* mutant mice.

The similar frequency of marrow $Lin^{-}Sca-1^{+}c-Kit^{+}$ cells in *Prg4* mutant vs. wildtype mice suggest that proteoglycan 4 is not a critical regulator of physiologic marrow HPC numbers. The finding that PTH increased $Lin^{-}Sca-1^{+}c-Kit^{+}$ cells more significantly in *Prg4* wildtype mice indicates that proteoglycan 4 supports PTH actions on HPC expansion. We speculate that while basal proteoglycan 4 expression does not impact HPC numbers, the PTH-induced increase in proteoglycan 4 supports HPC expansion, which is supported by recombinant HAPO administration studies (14;15). Daily intermittent subcutaneous injection of recombinant HAPO to normal mice has been shown to increase marrow HPC populations, including $CD34^{+}$, $c-Kit^{+}$, and $Sca-1^{+}$ cells (14). Since SDF-1 is a potent chemo-attractant that regulates the homing and mobilization of marrow HPC cells (46-49), the blunted ability of PTH to increase SDF-1

in the marrow of *Prg4* mutant mice may be linked to the blunted PTH expansion of marrow HPCs in *Prg4* mutant mice.

The finding that PTH increased bone marrow and calvaria IL-6 mRNA to a greater extent in *Prg4* mutant mice highlights that in the absence of *Prg4*, PTH differentially regulates the expression of PTH responsive genes, and may represent an attempt at compensation for the absence of proteoglycan 4. IL-6 supports HPC proliferation (43;50-52), and we have recently shown it to be a critical regulator of PTH actions in HPC expansion (13).

Since the calvarial organ is primarily composed of stromal and osteoblastic cells, the predominant PPR expressing cell population in the bone marrow, the more significantly increased PTH-induced IL-6 mRNA in *Prg4* mutant calvaria suggests that a stromal/osteoblastic cell is the target of PTH actions to modulate IL-6 and hematopoietic cells in the marrow of *Prg4* mutant mice. Studies demonstrating that single PTH injections regulated other established PTH responsive genes similarly in *Prg4* mutant vs. wildtype mice suggest that IL-6 signaling is specifically altered in *Prg4* mutant mice.

While it has been reported that the *Prg4* protein product MSF stimulates the growth of platelet forming cells *in vitro* (17-19), the similar number of peripheral blood platelets, frequency of marrow CD41⁺ cells, marrow megakaryocyte ploidy, and number of megakaryocytes per marrow area in *Prg4* mutant vs. wildtype mice suggests that proteoglycan 4 is not a critical regulator of physiologic megakaryopoiesis *in vivo*.

Prg4 actions on SDF-1 are unknown. The decreased SDF-1 protein levels in the marrow of *Prg4* mutant mice imply that proteoglycan 4 may interact with and increase the concentration of SDF-1 in the marrow. This is supported by prior studies, which have

demonstrated that SDF-1 binds cellular proteoglycans in the bone marrow (53-57). That a single PTH injection significantly increased SDF-1 mRNA in the marrow of wildtype but not mutant mice, may contribute to the blunted increase in marrow SDF-1 protein levels in PTH-treated *Prg4* mutant mice. Based on recent work demonstrating that intermittent PTH administration inhibits dipeptidyl peptidase-IV (DPP-IV) (58), an enzyme that functionally degrades SDF-1, an alternative explanation for the blunted PTH increase in marrow SDF-1 is that PTH actions protecting marrow SDF-1 from enzymatic degradation are decreased in *Prg4* mutant mice.

This investigation of *Prg4* actions on hematopoiesis revealed that proteoglycan 4 regulates marrow SDF-1 levels, immune cell populations, and PTH actions increasing marrow SDF-1 and HPCs. SDF-1 is a candidate regulator of proteoglycan 4 actions on hematopoiesis. Contrary to prior studies in which proteoglycan 4 was administered, characterization of the *Prg4* mutant mouse model demonstrates that basal proteoglycan 4 expression does not impact megakaryopoiesis or HPC frequency. While CACP patients have not been reported to spontaneously develop hematological abnormalities, we are unaware of any studies that characterize their bone marrow cell populations. The present study reveals that loss-of-function mutations in *Prg4* may result in altered immune cell populations which could have implications for immune response. In summary, proteoglycan 4 is a novel immunomodulatory factor regulating physiologic immune cell populations and PTH actions on hematopoiesis.

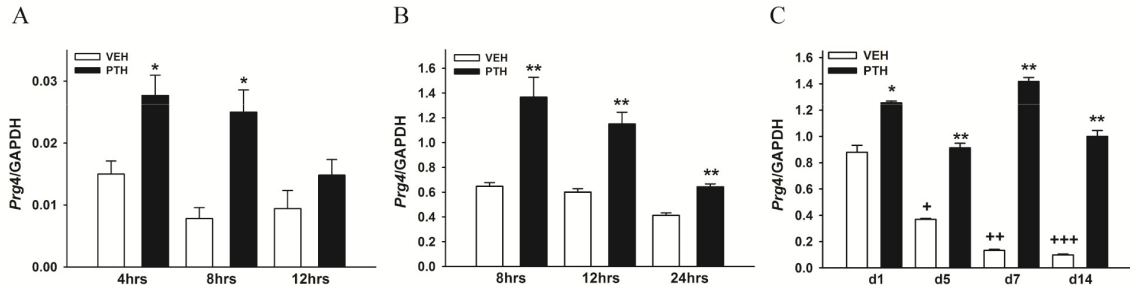


Figure 2.1. PTH regulation of *Prg4* mRNA. **A:** Sixteen week old C57BL6 wildtype mice were administered a single subcutaneous injection of PTH (1-34) (1 μ g/g) or vehicle (VEH) (0.9% NaCl) control, sacrificed 4, 8, or 12 hours (hrs) later, and bone marrow harvested for gene expression analysis (n=5/gp). * p<0.05 vs. VEH. **B:** C57BL6 wildtype mice derived calvarial osteoblast cultures were administered a single PTH (10 nM) or vehicle (VEH) (4mM HCl/0.1%BSA) treatment, and harvested 8, 12, or 24 hrs later for gene expression analysis (n=7/gp). ** p<0.01 vs. VEH. **C:** C57BL6 wildtype mice derived calvarial osteoblast cultures, 1, 5, 7, and 14 days after plating, were administered a single PTH (10 nM) or vehicle (VEH) (4mM HCl/0.1%BSA) treatment, and harvested 4 hrs later for gene expression analysis (n=3/gp). * p<0.05: PTH vs. VEH; ** p<0.001: PTH vs. VEH; +p<0.01: d5-VEH vs. d1-VEH; ++p<0.001: d7-VEH vs. d5-VEH; +++p<0.05: d14-VEH vs. d7-VEH. In each case, RNA was isolated and quantitative real-time PCR performed to assess *Prg4* mRNA expression (standardized to GAPDH levels). Relative quantification of data generated was determined using the standard curve method. Data is expressed as mean \pm SEM.

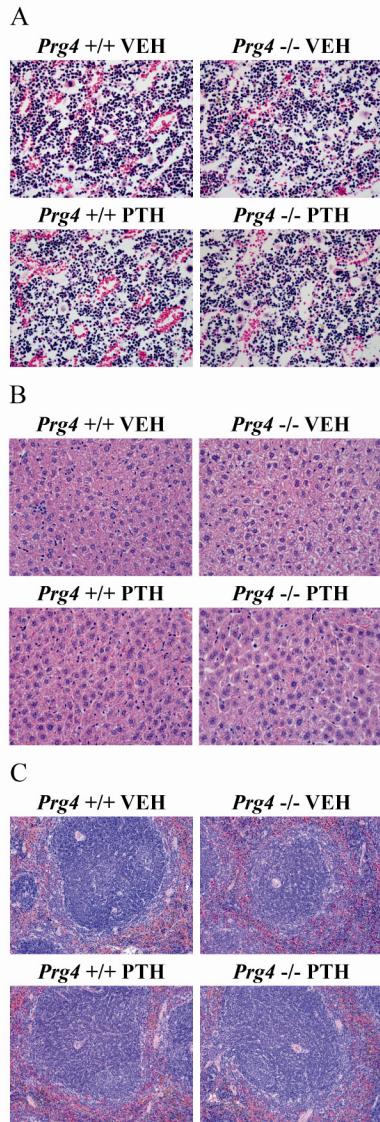


Figure 2.2. Histological assessment of bone marrow, liver, and spleen. **A-C:** *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 µg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16-22 weeks. Tibia, liver, and spleen were isolated from twenty-two week old *Prg4* -/- and +/+ mice for histological evaluation. Representative images of hematoxylin & eosin (H&E) stained (**A**) proximal tibia bone marrow sections (40X) ($n \geq 10/\text{gp}$), (**B**) liver sections (40X) ($n \geq 5/\text{gp}$), and (**C**) spleen sections (20X) ($n \geq 5/\text{gp}$).

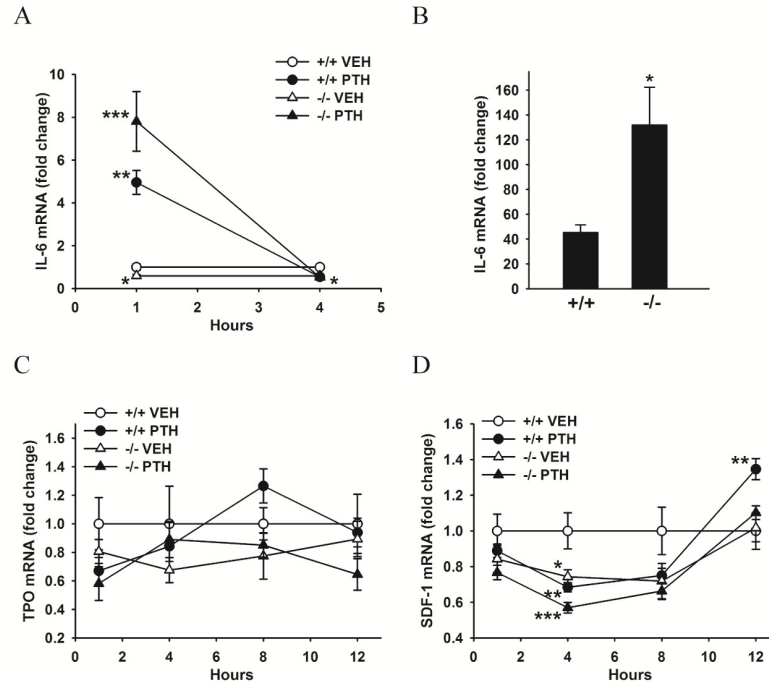


Figure 2.3. PTH regulation of gene expression. **A,C,D:** Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered a single subcutaneous injection of PTH (1-34) (1 μ g/g) or vehicle (VEH) (0.9% NaCl) control, sacrificed 1, 4, 8, or 12 hours later, and bone marrow harvested for gene expression analysis (n=5/gp). RNA was isolated and quantitative real-time PCR was performed to assess; **(A)** interleukin-6 (IL-6), **(C)** thrombopoietin (TPO), and **(D)** stromal cell-derived factor-1 (SDF-1) mRNA expression (standardized to GAPDH levels). Relative quantification of data was determined using the comparative CT method. **B:** Sixteen week old *Prg4* -/- and +/+ mice were administered a single subcutaneous injection of PTH (1-34) (1 μ g/g), sacrificed 0 (no treatment control) and 4 hours later, and calvaria harvested for gene expression analysis (n=5/gp). RNA was isolated and quantitative real-time PCR was performed to assess IL-6 mRNA expression (standardized to GAPDH levels). Values were expressed as treatment over control within treatment groups. **A:** Line graph represents marrow IL-6 mRNA fold change at 1 and 4 hours after PTH injection. * p<0.05: -/- VEH vs. +/+ VEH; ** p<0.001: +/+ PTH vs. +/+ VEH; *** p<0.001: -/- PTH vs. -/- VEH. **B:** Bar graph represents calvaria IL-6 mRNA fold change at 4 hours after PTH injection. * p<0.05: -/- PTH vs. +/+ PTH. **C:** Line graph represents marrow TPO mRNA fold change at 1, 4, 8, 12 hours after PTH injection. **D:** Line graph represents marrow SDF-1 mRNA fold change at 1, 4, 8, 12 hours after PTH injection. * p<0.05: -/- VEH vs. +/+ VEH; ** p<0.05: +/+ PTH vs. +/+ VEH; *** p<0.01: -/- PTH vs. -/- VEH. Data is expressed as mean \pm SEM.

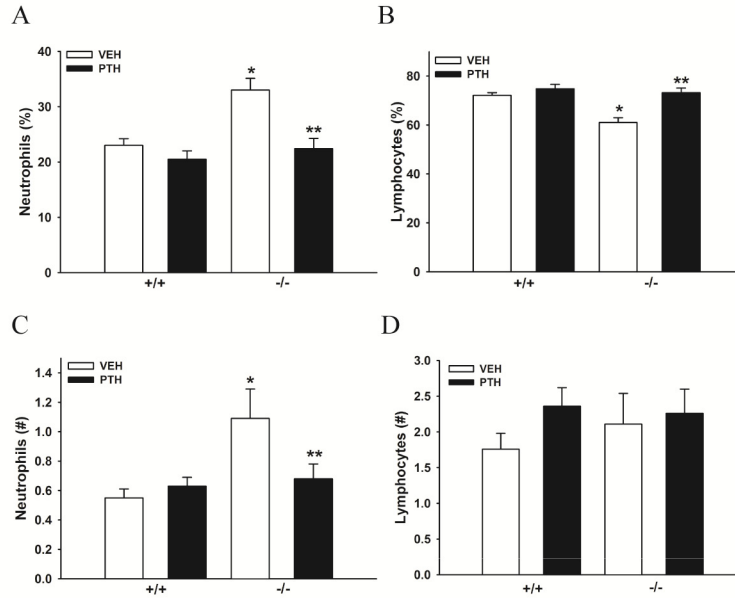


Figure 2.4. Peripheral blood cells. **A-D**: Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks, from age 16-22 weeks. Peripheral blood was isolated via cardiac puncture from twenty-two week old *Prg4* -/- and +/+ mice and complete blood count analysis (automated differential) was performed to assess peripheral blood cell populations ($n \geq 10$ /gp). **A**: Bar graph represents the percentage of neutrophils. * $p < 0.001$ vs. +/+ VEH; ** $p < 0.01$ vs. -/- VEH. **B**: Bar graph represents the percentage of lymphocytes. * $p < 0.001$ vs. +/+ VEH; ** $p < 0.001$ vs. -/- VEH. **C**: Bar graph represents the number of neutrophils. * $p < 0.01$ vs. +/+ VEH; ** $p < 0.05$ vs. -/- VEH. **D**: Bar graph represents the number of lymphocytes. Data is expressed as mean \pm SEM.

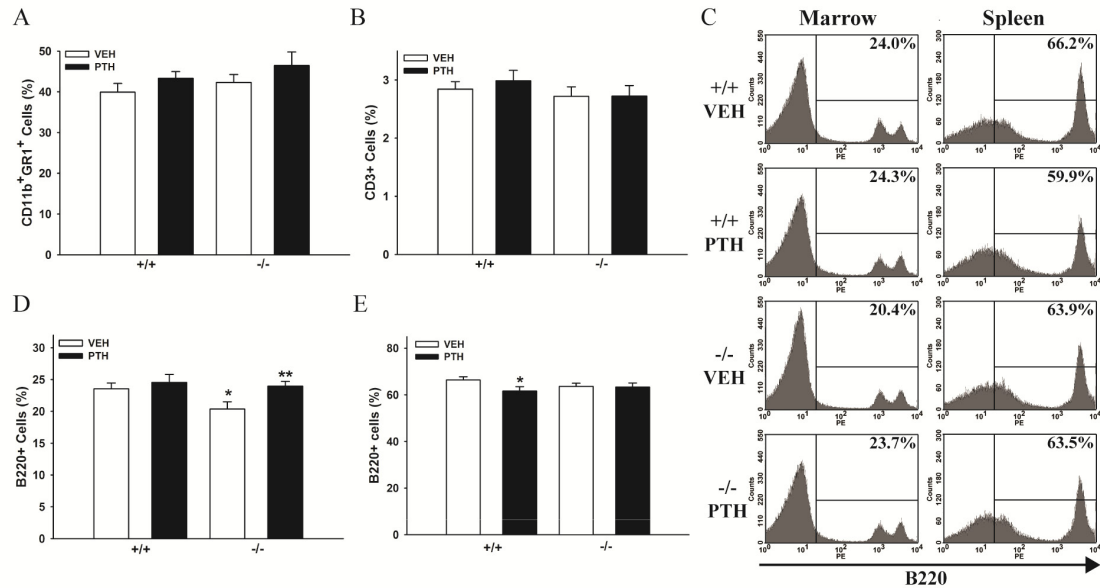


Figure 2.5. Bone marrow immune cells. **A-D:** *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16-22 weeks. Twenty-two week old *Prg4* -/- and +/+ mice were sacrificed and femur bone marrow cells were isolated for flow cytometric analysis of; **(A)** CD11b⁺GR1⁺ (immature myeloid) cells ($n \geq 9$ /gp) **(B)** CD3⁺ (T-lymphocytic) cells ($n \geq 9$ /gp), and **(C and D)** B220⁺ (B-lymphocytic) cells ($n \geq 9$ /gp). **C and E:** Spleen cells were isolated for flow cytometric analysis of B220⁺ (B-lymphocytic) cells ($n \geq 10$ /gp). **C:** Representative histogram images of marrow B220⁺ cells and spleen B220⁺ cells. Bar graphs represent the **(A)** percentage of CD11b⁺GR1⁺ cells out of total marrow cells, **(B)** the percentage of CD3⁺ cells out of total marrow cells, **(D)** the percentage of B220⁺ cells out of total marrow cells, **(E)** and the percentage of B220⁺ cells out of total spleen cells. * $p < 0.05$ vs. +/+ VEH; ** $p < 0.05$ vs. -/- VEH. Data is expressed as mean \pm SEM.

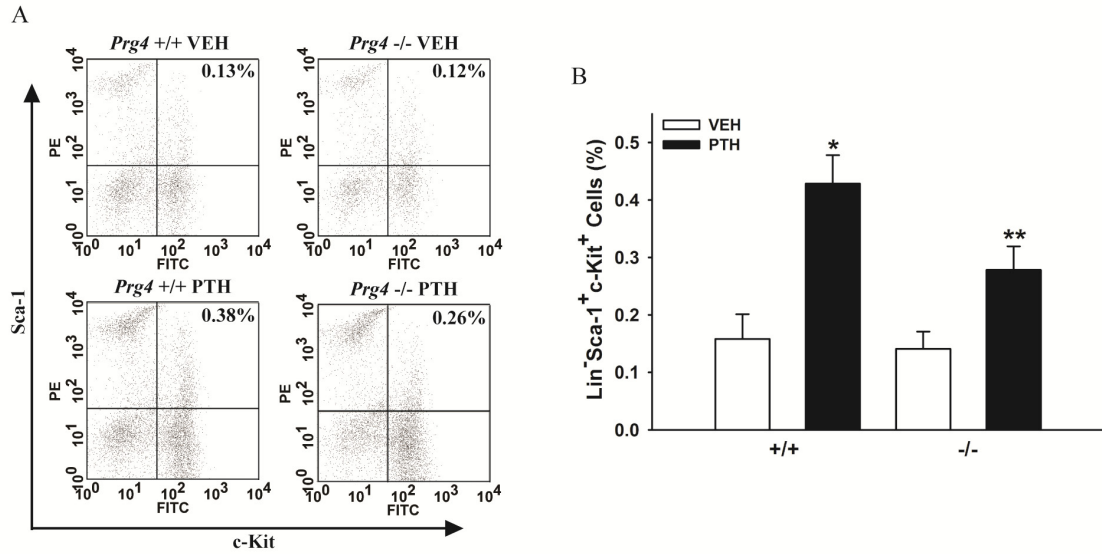


Figure 2.6. Bone marrow hematopoietic progenitor cells. **A** and **B**: *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16-22 weeks. Femur bone marrow cells were isolated from twenty-two week old *Prg4* -/- and +/+ mice for flow cytometric analysis of Lin⁻Sca-1⁺c-Kit⁺ (hematopoietic progenitor) cells (n \geq 6/gp). **A**: Representative dot plots of FITC/c-Kit (x-axis) vs. PE/Sca-1 (y-axis) (gated for Lin⁻ cell population). **B**: Bar graph represents the percentage of Lin⁻Sca-1⁺c-Kit⁺ cells out of total marrow cells. *p<0.01 vs. +/+ VEH; **p<0.05 vs. -/- VEH & +/+ PTH. Data is expressed as mean \pm SEM.

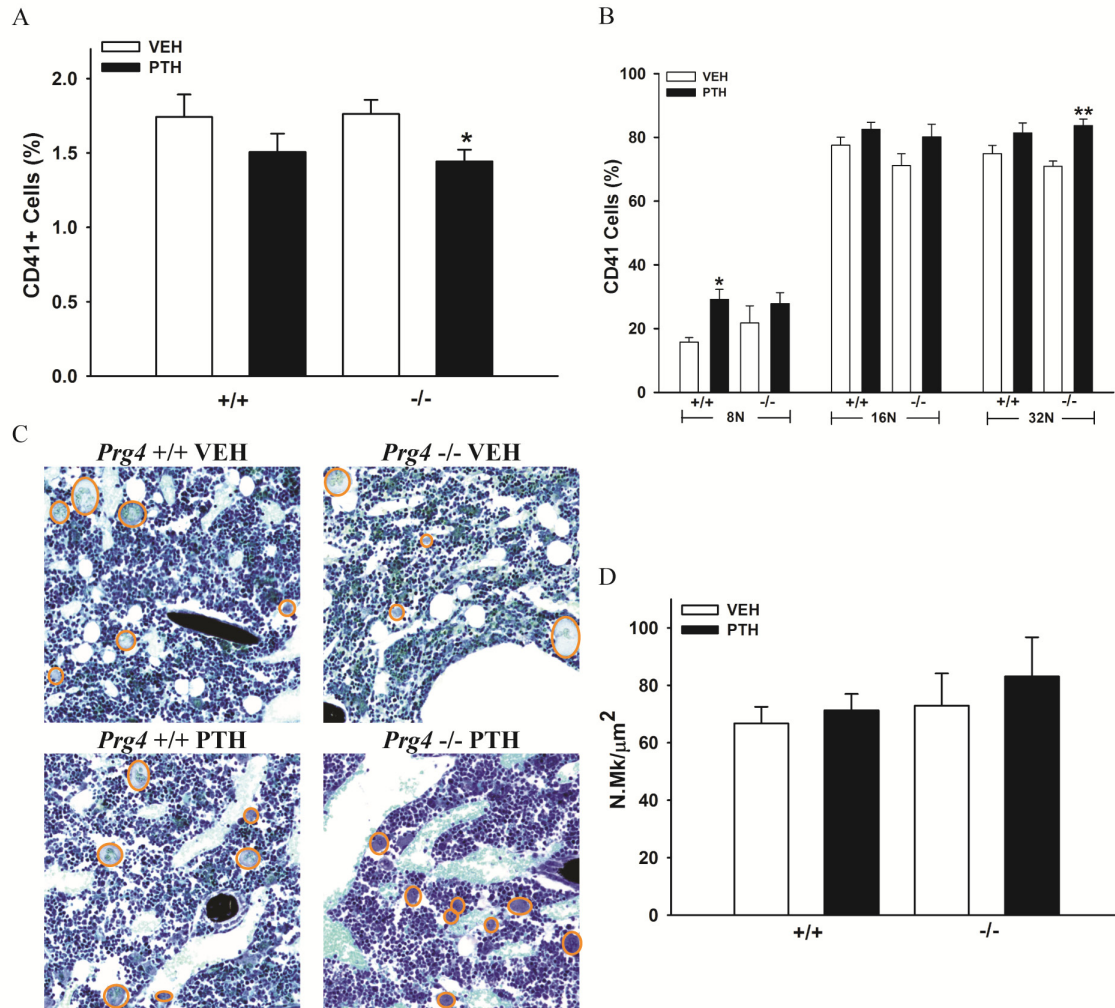


Figure 2.7. Bone marrow megakaryopoietic cells. **A-D:** *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16-22 weeks. **A** and **B:** Flow cytometric analysis of CD41+ (megakaryopoietic) cells in femur bone marrow from twenty-two week old *Prg4* -/- and +/+ mice (n≥8/gp). **A:** Bar graph represents the percentage of CD41+ cells out of total marrow cells. *p<0.05 vs. -/- VEH. **B:** Flow cytometric analysis of megakaryocyte ploidy from bone marrow of twenty-two week old *Prg4* -/- and +/+ mice (n≥7/gp). Bar graphs represent the percentage of CD41+ cells out of total cells within 8N, 16N, 32N ploidy classes. *p<0.001 vs. +/+ VEH; **p<0.001 vs. -/- VEH. **C:** Representative images of von Kossa (with tetrachrome counterstain) stained proximal tibia sections from twenty-two week old *Prg4* -/- and +/+ mice, with megakaryocytes highlighted by **orange circles**. **D:** Bar graphs represent the number of megakaryocytes per marrow area (n≥9/gp). Data is expressed as mean ± SEM.

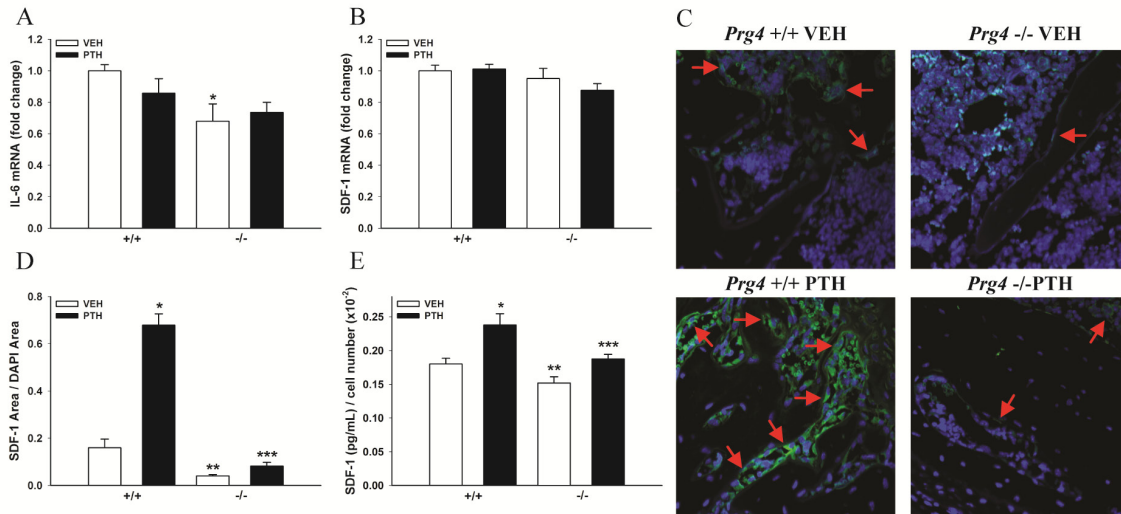


Figure 2.8. Bone marrow IL-6 and SDF-1 expression. **A-E:** *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16-22 weeks. **A** and **B:** Bone marrow was harvested from twenty-two week old *Prg4* -/- and +/+ mice (n=5/gp) for quantitative real-time PCR analysis of, **(A)** interleukin-6 (IL-6) and **(B)** stromal cell-derived factor-1 (SDF-1) mRNA expression (standardized to GAPDH levels). Relative quantification of data was carried out using the comparative CT method. **A:** Bar graph represents marrow IL-6 mRNA expression. *p<0.05 vs. +/+ VEH. **B:** Bar graph represents marrow SDF-1 mRNA expression. **C** and **D:** SDF-1 immunofluorescence (IF) analysis was carried out in twenty-two week proximal tibia (secondary spongiosa) sections (n=6/gp). **C:** Representative SDF-1 IF labeled proximal tibia sections (SDF-1/green and DAPI/blue overlay) (60X), with **red arrows** indicating selected SDF-1+ stromal/osteoblastic cells. **D:** Bar graph represents the SDF-1 area / DAPI area. *p<0.001 vs. +/+ VEH; **p<0.01 vs. +/+ VEH; ***p<0.05 vs. -/- VEH & +/+ PTH. **E:** ELISA analysis of bone marrow SDF-1 levels in twenty-two week old *Prg4* mice. Bar graph represents marrow SDF-1 (pg/mL) normalized to marrow cell numbers (n \geq 5/gp). *p<0.05: vs. +/+ VEH; **p<0.05: vs. +/+ VEH; ***p<0.05: vs. -/- VEH & +/+ PTH. Data is expressed as mean \pm SEM.

<i>Protein Product</i>	<i>Isolation</i>	<i>Expression</i>	<i>Known Functions</i>
Lubricin (21)	Bovine synovial fluids	Synoviocytes	Boundary lubrication of synovial joints
Superficial Zone Protein (SZP) (22)	Bovine superficial zone articular cartilage explant culture media	Superficial zone articular chondrocytes	Protects superficial zone articular cartilage
Hemangiopoietin (HAPO) (14)	Urine of patients afflicted by aplastic anemia	Unknown	Supports hematopoietic and endothelial progenitor cells
Megakaryocyte Stimulating Factor (MSF) (17)	Urine of bone marrow transfer patients afflicted by acute thrombocytopenia	Unknown	Regulates megakaryopoiesis

Table 2.1. *Prg4* protein products.

	+/+ VEH	+/+ PTH	-/- VEH	-/- PTH
White blood cells (K/μL)	2.43 \pm 0.28	3.13 \pm 0.32	2.92 \pm 0.60	3.08 \pm 0.44
Neutrophils (%)	23.0 \pm 1.21	20.5 \pm 1.51	***33.0 \pm 2.11	^{¶¶} 22.4 \pm 1.87
Neutrophils (#)	0.55 \pm 0.06	0.63 \pm 0.06	**1.09 \pm 0.20	[¶] 0.68 \pm 0.10
Lymphocytes (%)	72.1 \pm 1.11	74.8 \pm 1.83	***61.0 \pm 1.96	^{¶¶¶} 73.2 \pm 1.92
Lymphocytes (#)	1.76 \pm 0.22	2.36 \pm 0.26	2.11 \pm 0.43	2.26 \pm 0.34
Monocytes (%)	3.49 \pm 0.30	2.88 \pm 0.34	4.12 \pm 0.60	3.19 \pm 0.44
Monocytes (#)	0.08 \pm 0.01	0.09 \pm 0.02	0.13 \pm 0.04	0.10 \pm 0.02
Eosinophils (%)	0.96 \pm 0.24	1.41 \pm 0.29	1.34 \pm 0.31	0.98 \pm 0.29
Eosinophils (#)	0.02 \pm 0.01	0.04 \pm 0.01	0.03 \pm 0.01	0.03 \pm 0.01
Basophils (%)	0.45 \pm 0.12	0.48 \pm 0.09	0.51 \pm 0.19	0.26 \pm 0.07
Basophils (#)	0.01 \pm 0.00	0.01 \pm 0.00	0.01 \pm 0.00	0.01 \pm 0.00
Red blood cells (M/μL)	9.58 \pm 0.13	9.09 \pm 0.17	9.45 \pm 0.40	9.43 \pm 0.24
Hemoglobin (g/dL)	12.9 \pm 0.18	12.3 \pm 0.18	12.7 \pm 0.57	12.0 \pm 0.29
Hematocrit (%)	43.8 \pm 0.58	41.6 \pm 0.83	43.3 \pm 1.94	43.2 \pm 1.04
Platelets (K/ μ L)	774 \pm 47.1	784 \pm 25.8	712 \pm 65.4	701 \pm 69.5

Table 2.2. Peripheral blood cells. Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks, from age 16-22 weeks. Peripheral blood was isolated via cardiac puncture from twenty-two week old *Prg4* -/- and +/+ mice and complete blood count analysis (automated differential) was performed to assess peripheral blood cell populations (n \geq 10/gp). ** p<0.01 vs. +/+ VEH; *** p<0.001 vs. +/+ VEH; [¶]p<0.05 vs. -/- VEH; ^{¶¶}p<0.01 vs. -/- VEH; ^{¶¶¶}p<0.001 vs. -/- VEH.

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CHAPTER 3

PROTEOGLYCAN 4: A DYNAMIC REGULATOR OF SKELETOGENESIS AND PTH SKELETAL ANABOLISM

Abstract

Proteoglycan 4 (*Prg4*), known for its lubricating and protective actions in joints, is a strong candidate regulator of skeletal homeostasis and PTH anabolism. Proteoglycan 4 is a PTH responsive gene in bone and liver. *Prg4* null mutant mice were used to investigate the impact of proteoglycan 4 on skeletal development, remodeling, and PTH anabolic actions. Young *Prg4* mutant and wildtype mice were administered intermittent PTH (1-34) or vehicle daily from day 4-21. Young *Prg4* mutant mice had decreased growth plate hypertrophic zones, trabecular bone, and serum bone formation markers versus wildtype; but responded with a similar anabolic response to PTH. Adult *Prg4* mutant and wildtype mice were administered intermittent PTH (1-34) or vehicle daily from 16-22 weeks. Adult *Prg4* mutant mice had decreased trabecular and cortical bone, and blunted PTH-mediated increases in bone. Joint range of motion and animal mobility were lower in adult *Prg4* mutant versus wildtype mice. Adult *Prg4* mutant mice had decreased marrow and liver FGF-2 mRNA and reduced serum FGF-2, which were normalized by PTH. A single dose of PTH decreased the PTH/PTHrP receptor (PPR), and increased *Prg4* and FGF-2 to a similar extent in liver and bone. Proteoglycan 4

supports endochondral bone formation and the attainment of peak trabecular bone mass, and appears to support skeletal homeostasis indirectly by protecting joint function. Bone and liver derived FGF-2 likely regulate proteoglycan 4 actions supporting trabeculae formation. Blunted PTH anabolic responses in adult *Prg4* mutant mice are associated with altered biomechanical impact secondary to joint failure.

Introduction

Parathyroid hormone (PTH) has catabolic and anabolic actions in bone, depending on the mode of administration. Continuous PTH administration induces bone resorption, whereas intermittent PTH administration stimulates bone formation (1). Once daily teriparatide (PTH 1-34) injection is currently the only FDA approved anabolic agent for the treatment of osteoporosis, and is under clinical investigation for the treatment of localized osseous defects (2;3). While intermittent PTH (1-34) has proven bone forming actions, the mechanisms mediating these anabolic effects are poorly understood.

Osteoblasts and stromal cells are the predominant cells in bone that express the PTH/PTH-related protein (PTHrP) receptor (PPR). PTH signaling in osteoblastic cells has been shown to regulate the expression of growth factors critical for PTH induced anabolic actions in bone (4;5). Most notably, PTH anabolic actions have been linked to insulin-like growth factor I (IGF-I) and basic fibroblast growth factor 2 (FGF-2) (6-8). PTH rapidly up-regulates FGF-2 mRNA in cultured osteoblastic cells (4), and intermittent PTH administration increases IGF-I mRNA in bone *in vivo* (5). IGF-I and FGF-2 mutant mice have severely blunted to absent anabolic responses to PTH (6-8).

Proteoglycan 4 (*Prg4*), a novel PTH responsive gene in bone (9) (Novince et al., manuscript submitted), is a strong candidate regulator of the anabolic actions of PTH. The *Prg4* gene encodes an approximately 345-kD proteoglycan, consisting of 1,404 amino acids spanning 12 exons. *Prg4* is expressed in skeletal and non-skeletal tissues, with highest levels of expression in articular joints, bone, and liver (10;11). The four isolated *Prg4* protein products are secreted glycoproteins, which have been implicated in the protection of articular joints, expansion of hematopoietic progenitor cells, and regulation of megakaryopoiesis. A proteoglycan 4 receptor has not been identified (11;12).

Loss-of-function mutations in *PRG4* result in the human autosomal recessive disorder camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, which is primarily characterized by precocious joint failure (13). Similar to humans presenting with the CACP syndrome, the *Prg4* mutant mouse is afflicted by early onset joint arthropathy (11). Of interest, osteopenia has been noted in microradiographs of joints from *Prg4* mutant mice (11). While studies have demonstrated that *Prg4* is endogenously expressed in bone (10) and *Prg4* is a PTH responsive gene in the bone marrow *in vivo* and isolated osteoblastic cells *in vitro*, there have been no reported investigations of the actions of proteoglycan 4 in bone. The purpose of this study was to investigate the role of proteoglycan 4 as a regulator of skeletal development, remodeling, and PTH anabolic actions. This investigation of the *Prg4* mutant mouse model provides for the first study of proteoglycan 4 actions in bone.

Materials and Methods

***C57BL6* wildtype mice**

Long bone (femur and tibia freed of soft tissue), calvaria, bone marrow (flushed from a femur and tibia), and whole liver were harvested from untreated sixteen week old *C57BL6* wildtype mice for gene expression analyses. In a PTH administration experimental protocol, sixteen week old *C57BL6* wildtype mice were administered a single subcutaneous injection of recombinant human PTH (1-34) (1 µg/g) (Bachem, Torrence, CA) or vehicle (0.9% NaCl), sacrificed 1, 4, 8, or 12 hours later, and whole liver and calvaria were harvested for gene expression analyses. All animal studies were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA), and animals were maintained in accordance with approved UCUCA research protocols.

***Prg4* mutant (-/-) mice**

Prg4 mutant (-/-) mice, generated by homologous recombination in 129Sv/Ev-derived embryonic stem cells, were generously provided by Matthew Warman (Harvard) (11). *Prg4* -/- mice were backcrossed from the 129Sv/Ev genetic background to the *C57BL6* genetic background. A PCR-based assay was used to genotype the mice as previously described (11).

Sixteen week old female *Prg4* -/- mice and wildtype (+/+) littermates were administered a single subcutaneous injection of recombinant human PTH (1-34) (1 µg/g) (Bachem) or vehicle (0.9% NaCl), sacrificed 0 (no treatment control), 1, 4, 8, or 12 hours

later, and bone marrow (flushed from a femur and tibia) and whole liver were harvested for gene expression analyses.

In an intermittent PTH administration experimental protocol, four day old *Prg4* *-/-* and *+/+* littermate mice were administered once daily subcutaneous injection of recombinant human PTH (1-34) (50 μ g/kg) (Bachem) or vehicle (0.9% NaCl) control for 17 days, from day 4-21. *Prg4* mice treated from day 4-21 are referred to here as “young” *Prg4* mice. In another intermittent PTH administration experimental protocol, sixteen week old *Prg4* *-/-* and *+/+* littermate mice were administered once daily subcutaneous injection of recombinant human PTH (1-34) (50 μ g/kg) (Bachem) or vehicle (0.9% NaCl) control for 6 weeks, from 16-22 weeks. *Prg4* mice treated from 16-22 weeks are referred to here as “adult” *Prg4* mice. Adult *Prg4* mice were administered an intraperitoneal injection of calcein (Sigma-Aldrich, St. Louis, MO) (20 mg/kg), dissolved in calcein buffer (0.15M NaCl, 2% NaHCO₃), 5 days and 2 days prior to sacrifice. Twenty-four hours following the final PTH injection, mice were sacrificed and tissues harvested for analyses.

Quantitative real-time PCR

Bone marrow was directly flushed from a femur and tibia with TRIzol reagent (Invitrogen, Carlsbad, CO). Long bone (femur and tibia freed of soft tissue), calvaria, and whole liver were flash frozen, pulverized, and homogenized in TRIzol reagent. In each case, RNA was isolated following manufacturer’s protocol, and total RNA was quantified. Double-stranded cDNA was synthesized from 1.0 μ g of RNA, using Random Hexamers (Applied Biosystems, Branchburg, NJ) and Multiscribe Reverse Transcriptase (Applied Biosystems). cDNA was amplified using the TaqMan Universal PCR Master

Mix (Applied Biosystems) with TaqMan gene expression specific primers-probes (Applied Biosystems) for proteoglycan-4 (*Prg4*), PTH/PTH-related protein receptor (PPR), insulin-like growth factor I (IGF-I), and basic fibroblast growth factor 2 (FGF-2). Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) was used as an endogenous control. Amplification was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative quantification of data was carried out using the standard curve method or the comparative CT method (14).

Histomorphometry

Tibias were fixed in 10% phosphate-buffered formalin for 48 hours at 4°C. Tibias from young mice were decalcified in 10% EDTA for 12 days at room temperature, and tibias from adult mice were decalcified in 10% EDTA for 21 days at room temperature. Proximal tibiae were embedded in paraffin, and 5 µm serial frontal sections were cut and stained. Hematoxylin & eosin (H&E) stain was performed in all proximal tibia sections. Growth plate height measurements were performed in H&E stained proximal tibia sections from young mice. Five measurements of the proliferative zone and the hypertrophic zone were carried out in the central two-thirds of the growth plate of each sample as described by Yakar et al. (15). Trabecular bone area (BA/TA) analysis was carried out in the secondary spongiosa of H&E stained proximal tibia sections from young and adult mice. Proximal tibia sections from adult mice were stained for tartrate resistant acid phosphatase (TRAP) using a commercial leukocyte acid phosphatase assay kit (Sigma-Aldrich). The number of TRAP+ multinucleated (three or more nuclei) cells per millimeter bone perimeter trabecular bone were quantified in the secondary spongiosa. Histomorphometric analysis of H&E stained and TRAP stained proximal

tibia sections was performed using Image Pro Plus 5.1 software (Media Cybernetics, Silver Spring, MD) interfaced with a Nikon Eclipse E800 light/epifluorescent microscope.

Tibias from adult mice were fixed in 10% phosphate-buffered formalin for 48 hours at 4°C, dehydrated in graded ethanols and xylene, and embedded undecalcified in modified methacrylate. Serial frontal proximal tibia sections (4 and 8 μm) were cut with vertical bed microtomes (Leica/Jung 2065 and 2165 Bannockburn, IL) and affixed to slides pre-coated with 1% gelatin solution. 4 μm sections were stained by the von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA), and used for determining cellular endpoints. 8 μm unstained sections were used for analyzing calcein labels. Bone histomorphometric data were collected semi-automatically with a Nikon Eclipse E800 light/epifluorescent microscope and the OsteoMeasure/Trabecular Analysis System (OsteoMetrics Inc., Atlanta, GA).

Analysis of methacrylate embedded proximal tibia sections began 200 μm distal to the growth plate and 50 μm from endocortical surfaces, including a 1200 μm (width) X 800 μm (length) area. Number of osteoblasts per bone perimeter (N.Ob/Pm) and osteoid surface (OS/BS) were assessed in von Kossa stained (4 μm) sections. Fluorochrome (calcein) based indices of bone formation including bone formation rate (BFR/BS), mineral apposition rate (MAR), and mineralizing surface (MS/BS) were analyzed in unstained (8 μm) sections. Bone histomorphometry data are reported in accordance with standardized nomenclature (16).

Micro-CT

Femurs from young and adult mice were fixed in 10% phosphate-buffered formalin for 48 h at 4°C, and stored in 70% ethanol. Femurs were scanned in water at an 18- μm isotropic voxel resolution using eXplore Locus SP (GE Healthcare Pre-Clinical Imaging, London, ON, Canada), and calibrated three-dimensional images were reconstructed. Femur length was measured, and cortical bone morphology was assessed in a 1 mm segment of mid-diaphysis with GE Medical Systems MicroView v2.2 Advanced Bone Analysis Application software (GE Healthcare Pre-Clinical Imaging). The center plane of the 1 mm segment was defined as the midpoint between the most lateral point of the third trochanter and the most proximal point of the distal epiphyseal growth plate. Cortical bone morphometric variables analyzed included total area (Tt.Ar), cortical area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th), marrow area (Ma.Ar), endocortical perimeter (Ec.Pm), periosteal perimeter (Ps.Pm), and bone mineral density (BMD). Femoral trabecular bone morphology and micro-architecture were analyzed using the stereology function of GE Medical Systems MicroView v2.2 Advanced Bone Analysis Application software. Transverse CT slices were analyzed beginning 360 μm proximal to the distal growth plate and extending 1.98 mm proximally. Trabecular bone morphometric variables analyzed included bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular bone mineral density (Tb.BMD). Fixed thresholds of 1200 and 2000 Hounsfield Units for trabecular bone and cortical bone, respectively, was used to discriminate mineralized tissue. For samples from young mice, the location and

length of the region of interest was adjusted in proportion to femoral length. Micro-CT data are reported in accordance with Bouxsein et al. (17).

Bone marrow stromal cell (BMSC) in vitro assays

Femur and tibia whole bone marrow was isolated from untreated sixteen week old *Prg4* ^{-/-} and ^{+/+} littermate mice. Epiphyses were sectioned, a 22G ½ inch needle was gently rotated into the marrow cavity, and marrow cells were flushed with α -modified minimum essential medium (α -MEM) (Invitrogen). Bone marrow cells were disassociated, cells counts performed, and cells plated at 3,000,000 cells/cm² in 60 mm dishes in α -MEM supplemented with 20% fetal bovine serum (FBS) (HyClone, Provo, UT), 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 10 nM dexamethasone (Sigma-Aldrich).

Bone marrow stromal cells (BMSCs) (adherent cells) were isolated without passage at d5. Culture media and non-adherent cells were aspirated, and BMSCs were washed, trypsinized, and plated in α -MEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine for assays. Plating cell density was assay dependent. Each *in vitro* assay was carried out at least 3 times.

In a cell enumeration assay first passage BMSCs were plated in 24 well plates at 20,000 cells/cm². Cultures were carried out in triplicate and medium changed every other day. Cell counting was performed on day d1, 3, 5, 7, and 9 using trypan blue dye and a hemacytometer.

In a von Kossa mineralization assay first passage BMSCs were plated in 12 well plates at 100,000 cells/cm². Upon reaching confluency (d4), cultures (in triplicate) were treated with mineralization medium (50 μ g/ml ascorbic acid, 10mM β -glycerophosphate)

for 10d. At the end of the culture period, cells were fixed with 95% EtOH and stained with 5% AgNO₃ using the von Kossa method to detect mineralization (18).

Serum biochemical assays

Whole blood was collected by cardiac puncture at euthanasia, coagulated at room temperature for 30 min, centrifuged, and serum isolated. Serum was stored at -80°C until assayed. Enzyme immunoassays were used to measure the serum concentrations of tartrate-resistant acid phosphatase form 5b (TRAP5b) (Immunodiagnostic Systems, Fountain Hills, AZ), propeptide of type I procollagen (P1NP) (Immunodiagnostic Systems), osteocalcin (OCN) (Biomedical Technologies, Stoughton, MA), intact PTH (Immutopics, San Clemente, CA), fibroblast growth factor-23 (FGF-23) (C-Term) (Immutopics), insulin-like growth factor I (IGF-I) (R&D Systems, Minneapolis, MN), and basic fibroblast growth factor-2 (FGF-2) (R&D Systems). Serum calcium concentration was analyzed by a colorimetric assay (Pointe Scientific, Canton, MI). Assays were performed according to manufacturer's instructions.

Joint range of motion and animal mobility

Joint range of motion was assessed by measuring maximal hind paw extension. Adult mice were anesthetized with isoflurane, the right tibia was immobilized at 0° on a protractor, and maximal right hind paw extension was recorded.

Animal mobility was assessed by monitoring spontaneous exploratory behavior. Adult mice were placed in a 30 X 40 cm chamber, divided into twelve 10 X 10 cm grids. Chamber walls were 10 cm in height, and constructed with a firm transparent plastic material. Mice were placed in the chamber and videotaped via a Flip Video - HD camera (Cisco, San Jose, CA) for one minute. Videos were reviewed to score the number of

grids crossed and number of hindlimb stands. A hindlimb stand was enumerated when the animal braced its forepaws against the chamber wall and elevated its body with the hindpaws. The spontaneous exploratory behavior study was performed in the animal housing facility during the early morning (1:00-2:00 AM) in order to assess nocturnal activity. Mice were directly transferred from their housed cage into the twelve grid chamber. The twelve grid chamber was set up in a laminar flow hood positioned within reaching distance of all housed cages to minimize stressing the mice prior to assessing spontaneous exploratory behavior.

Statistical analysis

Unpaired *t*-tests were performed using GraphPad InStat Software (GraphPad Software, San Diego, CA). For comparison of +/+ PTH vs. -/- PTH samples, values were expressed as treatment over control prior to statistical analysis. Data are presented as mean \pm standard error of mean (SEM), and statistical significance is $p < 0.05$ or lower. Statistical analysis was carried out in consultation with the Center for Statistical Consultation and Research (CSCAR) at the University of Michigan.

Results

PTH regulates *Prg4* mRNA in bone and liver

Quantitative real-time PCR studies assessing *Prg4* mRNA expression in long bone, calvaria, bone marrow, and whole liver from untreated sixteen week old C57BL6 mice demonstrated that *Prg4* is expressed at highest levels in long bone and liver (**Figure 3.1A**). Based on the high relative expression of *Prg4* mRNA in liver (**Figure 3.1A**), and

since the liver expresses PPR (**Figure 3.1B**) (19;20), the impact of PTH on liver *Prg4* mRNA expression was assessed. A single subcutaneous injection of PTH (1-34) (1 µg/g) in sixteen week old C57BL6 wildtype mice significantly increased liver *Prg4* mRNA (4-fold) 4 hours after injection, and liver *Prg4* mRNA remained significantly up-regulated 8 and 12 hours after injection (**Figure 3.1C**). A single subcutaneous injection of PTH (1-34) also increased *Prg4* mRNA in calvariae (3-fold) 4 hours after injection (**Figure 3.1D**), demonstrating that PTH regulates *prg4* gene expression similarly in bone and liver. While the biologic role of PPR expression in the liver is unclear, the liver has been shown to support skeletal homeostasis (15;21;22) and PTH anabolic actions in the skeleton (23;24).

Decreased bone and blunted PTH anabolic actions in *Prg4* ^{-/-} mice

Histomorphometric analysis of trabecular bone area (BA/TA) in hematoxylin and eosin (H&E) stained proximal tibia sections revealed marginally less trabecular BA/TA in young *Prg4* ^{-/-} mice vs. ^{+/+} littermates (**Figure 3.2A,B**), whereas adult *Prg4* ^{-/-} mice had significantly decreased trabecular BA/TA relative to ^{+/+} littermates (**Figure 3.2B,C**). PTH similarly increased trabecular BA/TA in young *Prg4* ^{+/+} and ^{-/-} mice, by 25% and 34% respectively (**Figure 3.2A,B**). PTH treatment induced an 82% increase in trabecular BA/TA in adult *Prg4* ^{+/+} mice vs. a 21% increase in ^{-/-} littermates (p<0.001) (**Figure 3.2B,C**), demonstrating that PTH anabolic actions increasing trabecular BA/TA are blunted in adult *Prg4* ^{-/-} mice.

Micro-CT analysis of trabecular bone volume (BV/TV) in the distal femur showed similar findings as the histomorphometric analysis of proximal tibia. Young and adult *Prg4* ^{-/-} mice had significantly less trabecular BV/TV vs. age matched ^{+/+}

littermates (**Figure 3.2D-F**). The increase in trabecular BV/TV by PTH was similar in young *Prg4* *+/+* (130%) and *-/-* mice (149%) (**Figure 3.2D,E**). PTH treatment resulted in an 89% increase in trabecular BV/TV in adult *Prg4* *+/+* mice vs. a 44% increase in *-/-* littermates ($p \leq 0.10$) (**Figure 3.2E,F**), revealing that PTH actions increasing trabecular BV/TV are marginally reduced in adult *Prg4* *-/-* mice.

Further analysis of trabecular bone parameters in the distal femur demonstrated decreased trabecular bone mineral density (Tb.BMD), trabecular number (Tb.N), and increased trabecular separation (Tb.Sp) in both young and adult *Prg4* *-/-* mice (**Table 3.1**). Of interest, there was no difference in trabecular thickness (Tb.Th) in *Prg4* *-/-* vs. *+/+* mice. PTH increased Tb.BMD, Tb.Th, Tb.N, and decreased Tb.Sp similarly in young *Prg4* *-/-* vs. *+/+* mice. Interestingly, the anabolic actions of PTH increasing Tb.Th and Tb.BMD were significantly blunted in adult *Prg4* *-/-* vs. *+/+* mice. PTH induced a 39% increase in Tb.Th in adult *Prg4* *+/+* mice vs. a 13% increase in *-/-* mice ($p < 0.05$). Moreover, PTH treatment resulted in a 41% increase in Tb.BMD in adult *Prg4* *+/+* mice vs. a 16% increase in *-/-* mice ($p < 0.05$).

Micro-CT analysis of cortical bone in the femoral mid-shaft demonstrated no difference in cortical area fraction (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th), or cortical bone mineral density (BMD) in young *Prg4* *-/-* vs. *+/+* mice. Adult *Prg4* *-/-* mice had decreased Ct.Ar/Tt.Ar and Ct.Th vs. *+/+* littermates. Further analysis of cortical bone parameters revealed that adult *Prg4* *-/-* mice had increased cortical marrow area (Ma.Ar) and marginally increased endocortical perimeter (Ec.Pm) relative to *+/+* littermates. While PTH increased Ct.Ar/Tt.Ar, Ct.Th, and BMD in adult *Prg4* *+/+* mice, there was a lack of anabolic response to PTH in adult *Prg4* *-/-* mice (**Table 3.1**).

Altered growth plate in young *Prg4* ^{-/-} mice

Growth plate morphology and height were assessed in proximal tibias from young *Prg4* ^{-/-} and ^{+/+} mice to evaluate alterations in endochondral bone formation. The proliferative zone chondrocytes in *Prg4* ^{-/-} mice were arranged in short interrupted columns compared to long continuous columns in *Prg4* ^{+/+} mice (**Figure 3.3A**). While the proliferative zone height was similar in *Prg4* ^{-/-} vs. ^{+/+} mice, the hypertrophic zone height and total growth plate height were reduced in *Prg4* ^{-/-} vs. ^{+/+} mice (**Figure 3.3B-D**). PTH increased the hypertrophic zone height and total growth plate height similarly in *Prg4* ^{-/-} vs. ^{+/+} mice (**Figure 3.3C,D**). Measurements of femur length demonstrated no difference in longitudinal growth of long bones in young *Prg4* ^{-/-} vs. ^{+/+} mice (**Figure 3.3E**).

Reduced biochemical markers of bone formation in young *Prg4* ^{-/-} mice

Serum biochemical assays were used to investigate alterations in bone modeling and response to PTH in young *Prg4* ^{-/-} mice. Markers for bone formation, serum propeptide of type I procollagen (P1NP) and bone turnover, serum osteocalcin (OCN) were decreased in vehicle-treated *Prg4* ^{-/-} vs. ^{+/+} mice (**Figure 3.3F,G**). PTH did not alter serum P1NP in *Prg4* ^{-/-} or ^{+/+} mice (**Figure 3.3F**). PTH increased serum OCN in *Prg4* ^{-/-} mice, but did not effect serum OCN in ^{+/+} mice (**Figure 3.3G**). There was no difference in serum tartrate-resistant acid phosphatase 5b (TRAP5b), a marker for bone resorption, in control *Prg4* mice, and PTH increased serum TRAP5b in *Prg4* ^{-/-} vs. ^{+/+} mice similarly (**Figure 3.3H**).

Lack of differences in *Prg4* ^{-/-} bone marrow stromal cells (BMSCs)

Bone marrow stromal cell (BMSC) *in vitro* assays were performed to elucidate cell intrinsic differences in *Prg4* ^{-/-} stromal/osteoblastic cells. There was no difference in *Prg4* ^{-/-} vs. ^{+/+} BMSC numbers over time (**Figure 3.4A**), which suggests that BMSCs from *Prg4* ^{-/-} mice do not have alterations in proliferation or apoptosis. Similar mineralization was present in *Prg4* ^{-/-} vs. ^{+/+} BMSC cultures (**Figure 3.4B,C**), which suggests that BMSCs from *Prg4* ^{-/-} mice do not have alterations in mineralization or differentiation.

Similar bone remodeling in adult *Prg4* ^{-/-} and ^{+/+} mice

In order to elucidate whether differences in bone cell numbers or activity mediate the osteopenic skeletal phenotype and blunted anabolic response to PTH in adult *Prg4* ^{-/-} mice, static cellular and dynamic histomorphometric analyses were performed in the secondary spongiosa of proximal tibias from adult *Prg4* mice (**Figure 3.5A-H**). There were no differences in numbers of osteoblasts per bone perimeter (N.Ob/B.Pm), osteoclastic TRAP⁺ cells per bone perimeter, osteoid surface (OS/BS), bone formation rates (BFR/BS), or mineral apposition rates (MAR) in control *Prg4* ^{-/-} vs. ^{+/+} mice, and PTH increased N.Ob/B.Pm, TRAP⁺ cells, OS/BS, BFR/BS, and MAR in *Prg4* ^{-/-} vs. ^{+/+} mice similarly (**Figure 3.5A-H**). These findings indicate that trabecular bone cell numbers, bone formation, and bone mineralization are unaffected by lack of proteoglycan 4 in the mature remodeling skeleton. Moreover, serum biochemical assays demonstrated no differences in markers for bone turnover or mineral homeostasis in adult *Prg4* ^{-/-} mice (**Figure 3.6A-F**).

Restricted joint range of motion and decreased mobility in adult *Prg4* ^{-/-} mice

Based on reports that adult *Prg4* ^{-/-} mice have failing articular joints (11) with significant loss of cartilage structure, stiffness, and frictional properties (25), we investigated changes in the joints of *Prg4* ^{-/-} mice which could contribute to the osteopenic skeletal phenotype and blunted PTH anabolic response in adult *Prg4* ^{-/-} mice. Maximal hind paw extension was assessed as a measure of joint range of motion (**Figure 3.7A,B**), and spontaneous exploratory behavior was monitored to evaluate animal mobility (**Figure 3.7C,D**). Maximal hind paw extension was limited to 115° in *Prg4* ^{-/-} mice compared to 170° in ^{+/+} mice (**Figure 3.7A,B**). The scoring of spontaneous exploratory behavior in a twelve grid chamber demonstrated that *Prg4* ^{-/-} mice crossed less grids and stood on their hindlimbs fewer times than ^{+/+} littermates (**Figure 3.7C,D**). The decreased joint range of motion and animal mobility demonstrated by adult *Prg4* ^{-/-} mice suggests that skeletal loading is altered in *Prg4* ^{-/-} mice.

Reduced liver IGF-I and FGF-2 and marrow FGF-2 mRNA normalized by PTH in *Prg4* ^{-/-} mice

Bone marrow and whole liver were isolated from adult *Prg4* mice to assess basal gene expression and the impact of six weeks daily PTH treatment on PPR, IGF-I, and FGF-2 mRNA expression. Marrow PPR mRNA was similar in control adult *Prg4* ^{-/-} and ^{+/+} mice (**Table 3.2**), which suggests that similar numbers of PPR expressing osteoblastic cells are present in the marrow of adult *Prg4* ^{-/-} and ^{+/+} mice. Liver PPR mRNA was decreased in control adult *Prg4* ^{-/-} vs. ^{+/+} mice. It has been reported that hepatocytes are the predominant PPR expressing cells in the liver (20).

IGF-I mRNA expression was assessed in marrow and liver since marrow derived IGF-I and liver derived IGF-I have been shown to independently support skeletal homeostasis (15;21;22) and PTH skeletal anabolism (23;24). Stromal/osteoblastic cells express IGF-I locally in the bone marrow mediating autocrine/paracrine signaling (26;27), and the liver secretes IGF-I into the circulation facilitating endocrine signaling in bone (28).

Unexpectedly, PTH significantly decreased marrow IGF-I mRNA in adult *Prg4* *+/+* mice and marginally decreased marrow IGF-I mRNA in adult *Prg4* *-/-* mice (**Table 3.2**). While it has been reported that intermittent PTH increases IGF-I mRNA expression in bone matrix devoid of bone marrow (5;29), we are unaware of prior studies that measured the effect of intermittent PTH on marrow IGF-I mRNA. Adult *Prg4* *-/-* mice had reduced liver IGF-I mRNA, and PTH normalized liver IGF-I mRNA in *Prg4* *-/-* mice to *+/+* levels (**Table 3.2**). The decreased liver IGF-I mRNA in control adult *Prg4* *-/-* mice suggests that liver *Prg4* may be important for IGF-I expression in the liver.

FGF-2 mRNA was assessed as another critical regulator of skeletal remodeling (30) and PTH anabolic actions (8;31). Adult *Prg4* *-/-* mice had lower marrow and liver FGF-2 mRNA than *+/+* mice (**Table 3.2**), which implies that *prg4* supports FGF-2 gene expression in marrow and liver. PTH increased marrow and liver FGF-2 mRNA in adult *Prg4* *-/-* mice to *+/+* levels (**Table 3.2**).

Reduced serum FGF-2 normalized by PTH in *Prg4* *-/-* mice

Serum IGF-I and FGF-2 were assayed in adult *Prg4* *-/-* and *+/+* mice to determine if alterations in *Prg4* *-/-* marrow and liver mRNA expression resulted in changes in the concentration of circulating proteins. There was no difference in serum IGF-I in adult

Prg4 $-/-$ and $+/+$ mice (**Table 3.3**), which suggests that the reduced liver IGF-I mRNA expression in *Prg4* $-/-$ mice is sufficient to support normal levels of circulating IGF-I. In contrast, adult *Prg4* $-/-$ mice had lower serum FGF-2 levels, and PTH increased serum FGF-2 in *Prg4* $-/-$ mice to $+/+$ levels (**Table 3.3**). The reduced serum FGF-2 correlated with decreased liver and marrow FGF-2 mRNA in adult *Prg4* $-/-$ mice, which were normalized to $+/+$ levels by PTH. While circulating IGF-I has been shown to be secreted primarily by the liver (75-80%) (28), the source of circulating FGF-2 is unclear. Based on quantitative real-time PCR demonstrating comparable FGF-2 mRNA expression in bone marrow and liver (**data not shown**), we speculate that decreased circulating FGF-2 levels in adult *Prg4* $-/-$ mice are attributed to both decreased marrow and liver FGF-2 mRNA expression.

PTH alters gene expression similarly in bone marrow and liver

Since PTH regulates *Prg4* mRNA expression similarly in bone and liver (**Figure 3.1C,D**), single PTH injection studies were used to elucidate whether *Prg4* regulates the expression of several critical PTH responsive osteogenic genes in bone and liver. PTH decreased marrow PPR mRNA at 1 hour and increased marrow PPR mRNA 8 hours after injection similarly in sixteen week old *Prg4* $-/-$ and $+/+$ mice (**Figure 3.8A**). PTH decreased liver PPR mRNA 4 hours after injection similarly in *Prg4* $-/-$ and $+/+$ mice (**Figure 3.8D**). The finding that PTH similarly down-regulates PPR mRNA in bone marrow and liver suggests that proteoglycan-4 does not modify PTH binding and signaling at the PPR receptor in bone or liver.

While intermittent PTH treatment has been reported to increase IGF-I mRNA expression in bone matrix *in vivo* (5;29), little is known regarding the temporal effects of

PTH on IGF-I mRNA expression. Marrow IGF-I mRNA was decreased 4 hours after PTH injection in *Prg4* *+/+* mice. Twelve hours following PTH injection, marrow IGF-I mRNA was significantly increased in *Prg4* *+/+* mice. PTH did not significantly alter marrow IGF-I mRNA in *Prg4* *-/-* or liver IGF-1 mRNA expression in *Prg4* *-/-* or *+/+* mice (**Figure 3.8B,E**).

Single PTH injection significantly increased marrow and liver FGF-2 mRNA in *Prg4* *-/-* and *+/+* mice at 1 hour (**Figure 3.8C,F**). These findings were consistent with prior studies that demonstrated FGF-2 is an early immediate PTH responsive gene in bone (4).

Consistent with control (vehicle-treated) adult *Prg4* *-/-* mice (**Table 3.2**), control (time 0 – no treatment control) sixteen week old *Prg4* *-/-* mice (**Figure 3.8A,B**) had similar marrow PPR and IGF-I mRNA levels compared to age matched *+/+* mice. While control adult *Prg4* *-/-* mice had decreased marrow FGF-2 mRNA (**Table 3.2**), marrow FGF-2 mRNA was similar in control sixteen week old *Prg4* *-/-* vs. *+/+* mice (**Figure 3.8C**). This finding indicates that decreased marrow FGF-2 mRNA in adult *Prg4* *-/-* mice is likely secondary to changes with age in the adult *Prg4* *-/-* mice.

Similar to control adult *Prg4* *-/-* mice (**Table 3.2**), control sixteen week old *Prg4* *-/-* mice (**Figure 3.8D-F**) had decreased liver PPR, IGF-I, and FGF-2 mRNA vs. age matched control *+/+* mice. These consistent gene expression findings in the liver of adult *Prg4* *-/-* mice and sixteen week old *Prg4* *-/-* mice indicate that liver *prg4* supports physiologic PPR, IGF-I, and FGF-2 gene expression in the liver.

Discussion

The four *Prg4* protein products; lubricin, superficial zone protein (SZP), hemangiopoietin (HAPO), and megakaryocyte stimulating factor (MSF) are secreted glycoproteins that have been implicated in the protection of articular joints, support of hematopoietic progenitor cells, and regulation of megakaryopoiesis. While studies report that proteoglycan 4 has diverse biologic actions, Proteoglycan 4 receptors have not been identified and receptor sites are unknown (11;12). Although most studies of *Prg4* have focused on the role of the *Prg4* gene product lubricin and joint function, we hypothesized that proteoglycan 4 also plays a role in skeletal development and remodeling based upon *Prg4* expression in bone (10) and the noted osteopenic phenotype in the *Prg4* mutant mouse (11). Furthermore, our findings that *Prg4* is a PTH responsive gene in bone lead us to speculate that proteoglycan 4 supports the anabolic actions of PTH in the skeleton.

Lubricin and SZP are expressed locally in the synovial joints by synoviocytes and superficial zone articular chondrocytes, having lubricating and protective effects (11;32). While the source of HAPO and MSF is unknown, based on studies that implicate HAPO and MSF in the regulation of hematopoiesis (12;33) and demonstration of high *Prg4* expression in liver and bone (10) (major sites of hematopoiesis), it is likely that both liver and bone act as considerable sources of proteoglycan 4. The novel finding that *Prg4* is a PTH responsive gene not only in bone but also liver implies that both marrow and liver derived proteoglycan 4 are candidate regulators of skeletal remodeling and the anabolic actions of PTH.

The finding that young *Prg4* mutant mice have decreased trabecular bone suggests that proteoglycan 4 plays a role in endochondral bone formation. The decreased height of the growth plate hypertrophic zone in young *Prg4* mutant mice suggests that proteoglycan 4 regulates growth plate chondrocyte maturation (34), which could have implications for endochondral cartilage ossification in developing long bone. Similar femoral length in young *Prg4* mutant and wildtype mice indicates that abnormalities in the growth plates of *Prg4* mutant mice do not impact long bone longitudinal growth.

While there were reduced numbers of trabeculae and increased trabecular separation in both young and adult *Prg4* mutant versus wildtype mice, there was no difference in trabecular thickness. This suggests that proteoglycan 4 functions to support the formation of distinct trabeculae during endochondral bone formation, but does not effect the remodeling of trabeculae. Based upon the findings that serum P1NP and OCN were decreased, and there were no differences in cortical bone or serum TRAP5b in young *Prg4* mutant mice, we speculate that decreased bone formation is associated with the reduced number of trabeculae in young *Prg4* mutant mice. Since BMSCs from *Prg4* mutant mice were normal, proteoglycan 4 actions supporting osteoblast mediated formation of trabeculae appear to be unique to the process of endochondral bone formation. The lack of difference in bone cell numbers or activity in the secondary spongiosa of adult *Prg4* mutant mice demonstrates that proteoglycan 4 is not a critical regulator of skeletal remodeling at trabecular sites in the mature skeleton. This finding suggests that the trabecular bone osteopenic phenotype in adult *Prg4* mutant mice is likely secondary to proteoglycan 4 actions supporting endochondral bone formation.

Based on studies characterizing the role of FGF-2 in skeletal development and remodeling, we speculate that the decreased marrow and serum FGF-2 levels may play a role in the trabecular bone osteopenia in *Prg4* mutant mice. Exogenous FGF-2 administration stimulates endosteal and endochondral bone formation in growing rats (35). In adult rats, FGF stimulates bone formation at endosteal and trabecular bone surfaces (36;37), and has been shown to induce the formation of de novo bone spicules within the marrow cavity (36). FGF-2 is expressed in differentiating growth plate chondrocytes, the centers of ossification, and the calcified matrix (38), which suggests that FGF-2 plays a role in the formation of trabeculae during endochondral bone formation. Bone marrow stromal/osteoblastic cells secrete FGF-2 (39;40) that signals to stromal/osteoblastic cells in an autocrine/paracrine manner (41;42). While the liver expresses FGF-2 (43;44), little is known regarding the role of the liver in secreting FGF-2 into the circulation, or the impact of circulating FGF-2 on the skeleton. Findings in the present study suggest that liver derived FGF-2 contributes to circulating levels which impact skeletogenesis.

Similarities in the skeleton of *Prg4* mutant and FGF-2 mutant mice suggest that FGF-2 is a candidate regulator of proteoglycan 4 actions supporting the formation of numbers of trabeculae. While neither *Prg4* mutant or FGF-2 mutant mice exhibit gross abnormalities in skeletal development or remodeling, both models have significantly reduced trabecular bone in the proximal tibia and distal femur sites that becomes more severe with age (30). Uniquely similar to the young and adult *Prg4* mutant mice, eighteen week old FGF-2 mutant mice have decreased numbers of trabeculae with increased trabecular separation, but no difference in trabecular thickness (30).

While there are no known studies investigating the osteogenic role of circulating FGF-2, based on studies demonstrating that circulating IGF-I has osteogenic effects separate from marrow IGF-I (15;21-24), we speculate that circulating FGF-2 may have an important role in skeletal homeostasis and PTH skeletal anabolism. Similar to the actions of PTH increasing serum FGF-2 levels in the osteopenic *Prg4* mutant mice, intermittent PTH has been reported to increase serum FGF-2 levels in osteoporotic patients (45). These data imply that the anabolic actions of PTH in the osteopenic/osteoporotic skeleton are mediated at least in part by an increase in circulating FGF-2.

Based on studies demonstrating that exogenous FGF-2 does not increase periosteal bone formation (35;46) and FGF-2 mutant mice do not have reported abnormalities in cortical bone (30), it does not appear that the osteopenia of cortical bone in *Prg4* $-/-$ mice is caused by the reduced FGF-2 levels. Since there were no differences in cortical bone in the femur of young *Prg4* mutant mice, the finding that cortical area fraction and cortical thickness were decreased in the femur of adult *Prg4* mutant mice indicates that proteoglycan 4 supports cortical bone homeostasis in the mature remodeling skeleton. The increased endocortical perimeter and marrow area associated with the decreased cortical bone thickness in the femur of adult *Prg4* mutant mice suggests that endocortical resorption is occurring at a more rapid rate than periosteal bone formation. Taking into consideration that adult *Prg4* mutant mice had decreased joint range of motion and animal mobility, it is likely that altered skeletal loading may account for the cortical bone osteopenic phenotype. Similar to the cortical bone osteopenia in adult *Prg4* mutant mice, rodent hindlimb immobilization studies have demonstrated that

cortical bone disease osteopenia is characterized by decreased cortical thickness, increased endocortical perimeter, and expansion of the marrow cavity (47;48). While we have proposed that the trabecular bone osteopenia in adult *Prg4* mutant mice is likely secondary to altered endochondral bone formation, it is possible that altered skeletal loading may contribute to the decreased trabecular bone in adult *Prg4* mutant mice.

While there have been controversial findings from investigations of PTH anabolic actions in rodent hindlimb immobilization models, studies demonstrating that loading is a critical regulator of PTH actions increasing trabecular and cortical bone (49;50) imply that the blunted PTH induced increase in bone in adult *Prg4* mutant mice may be due to compromised joint function leading to altered skeletal loading. PTH has been reported to restore lost trabecular bone in unloaded rats to the levels of non-treated loaded controls (49;50) which is similar to what was seen in the present study where PTH-treated adult *Prg4* mutant mice had similar trabecular bone levels as untreated wildtype mice. Concerning cortical bone, similar to the lack of a PTH increase in cortical bone in adult *Prg4* mutant mice, PTH does not significantly prevent cortical bone loss or restore lost cortical bone in unloaded rats compared to the levels of non-treated loaded controls (49;51). While there have been reports suggesting that the anabolic actions of PTH on trabecular bone are independent of the level of mechanical usage (52;53) findings from the current study of blunted PTH actions on trabecular bone in the compromised joint status of the *Prg4* mutant mice, suggest that physiologic skeletal loading is a critical regulator of PTH anabolic actions. This study of the effects of PTH in an animal model having precocious joint failure raises the question of the efficacy of PTH anabolic therapy in patients afflicted by arthropathic joint conditions that alter skeletal loading.

The novel finding that PTH regulates PPR, FGF-2, and *prg4* mRNA expression similarly in bone and liver suggests that the liver plays an important role in mediating the biologic actions of PTH. In conjunction with studies demonstrating that the anabolic actions of PTH are blunted in hepatocyte specific IGF-I knockout models (24;25), the present study and work by Mitnick et al. (54) which show that PTH regulates liver gene and protein expression, provide evidence that the liver supports PTH biologic actions. Historically, PTH research has focused on the role of bone as a mediator of PTH biologic actions. This focus is well grounded in knowledge that stromal/osteoblastic cells are predominant cells in the body expressing PPR, and PTH signaling in stromal/osteoblastic cells regulates the local expression of factors critical for the anabolic actions of PTH. Studies demonstrating that PPR is expressed at relatively high levels in the liver (20), the liver is a PTH responsive organ (54), and the liver supports the anabolic actions of PTH (23;24) emphasize the need for investigations of the role of the liver as a mediator of PTH biologic actions.

This original investigation of *Prg4* actions in the skeleton revealed that proteoglycan 4 is an important regulator of skeletal development, remodeling, and PTH anabolic actions. Proteoglycan 4 supports endochondral bone formation and the attainment of peak trabecular bone mass in the developing skeleton. In the mature remodeling skeleton proteoglycan 4 appears to indirectly support skeletal homeostasis and PTH anabolic actions by protecting joint function. Bone and liver derived FGF-2 are candidate regulators of proteoglycan 4 actions supporting the formation of trabeculae numbers. In summary, proteoglycan 4 is a dynamic factor supporting skeletogenesis and PTH skeletal anabolism via actions regulating trabecular bone formation and protecting

joint biomechanics. Finally, in addition to well characterized actions in osteoblasts, PTH likely impacts skeletogenesis via actions in the liver.

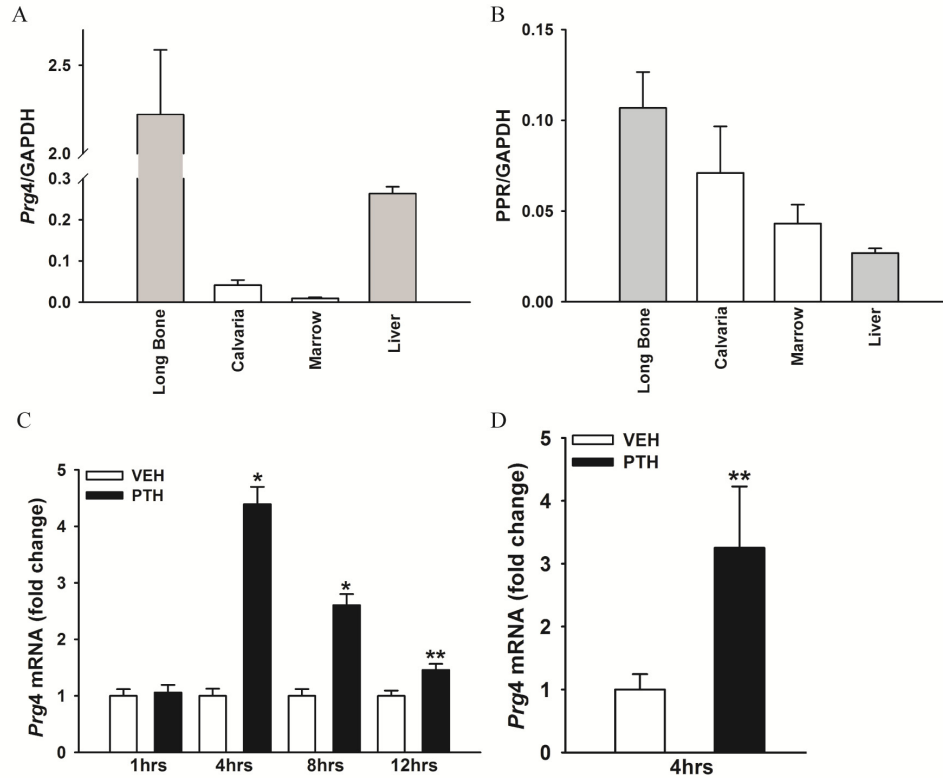


Figure 3.1. PTH regulation of *Prg4* mRNA. **A and B:** Untreated sixteen week old C57BL6 wildtype mice were sacrificed, and long bone, calvaria, bone marrow, and whole liver were harvested for gene expression analysis ($n=3/\text{gp}$). RNA was isolated and quantitative real-time PCR was performed to assess; **(A)** proteoglycan-4 (*Prg4*) mRNA, and **(B)** PTH/PTHrP receptor (PPR) mRNA expression (standardized to GAPDH levels). Relative quantification of data was determined using the standard curve method. **C and D:** Sixteen week old C57BL6 wildtype mice were administered a single subcutaneous injection of PTH (1-34) (1 $\mu\text{g/g}$) or vehicle (VEH) (0.9% NaCl) control, sacrificed 1, 4, 8, or 12 hrs later, and whole liver and calvaria were harvested for gene expression analysis ($n\geq 5/\text{gp}$). RNA was isolated and quantitative real-time PCR was performed to assess *Prg4* mRNA expression (standardized to GAPDH levels) in; **(C)** liver, and **(D)** calvaria. Relative quantification of data generated was carried out using the comparative CT method. * $p<0.001$; ** $p<0.05$ vs. time matched VEH. Data is expressed as mean \pm SEM.

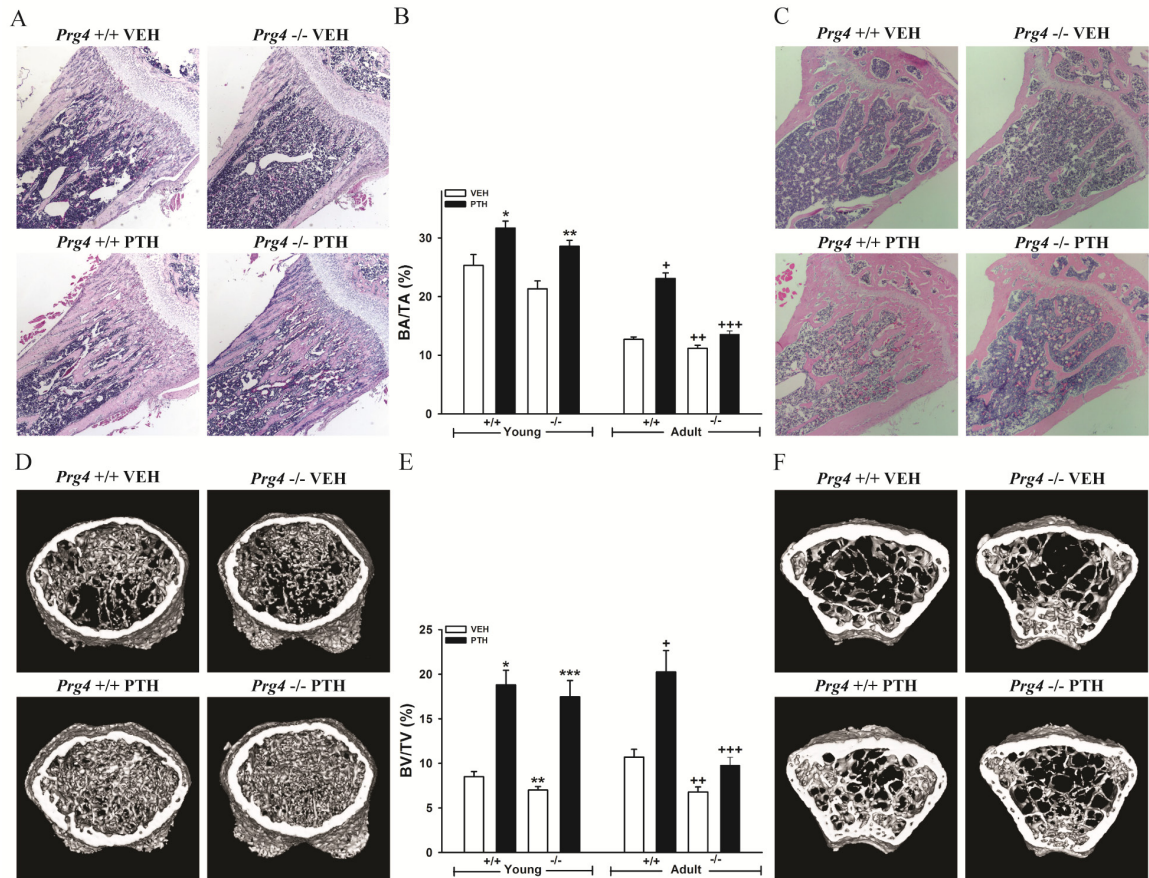


Figure 3.2. Trabecular bone area and volume analysis. **A,B,D,E:** Four day old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 $\mu\text{g}/\text{kg}$) or vehicle (VEH) (0.9% NaCl) subcutaneous injection daily for 17 days (“young” mice). **B,C,E,F:** Sixteen week old *Prg4* -/- and +/+ mice were administered intermittent PTH (1-34) (50 $\mu\text{g}/\text{kg}$) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks (“adult” mice). Femur and tibia were harvested at time of sacrifice. **A-C:** Histomorphometric analysis of trabecular bone area (BA/TA) in proximal tibia (secondary spongiosa) of young ($n \geq 11/\text{gp}$) and adult ($n \geq 13/\text{gp}$) mice. Representative images (4X) of H&E stained proximal tibial sections from, (**A**) young and (**C**) adult mice. **B:** trabecular BA/TA in the proximal tibia (secondary spongiosa). * $p < 0.01$: vs. +/+ VEH; ** $p < 0.001$: vs. -/- VEH; + $p < 0.001$: vs. +/+ VEH; ++ $p < 0.05$: vs. +/+ VEH; +++ $p < 0.01$: vs. -/- VEH & +/+ PTH. **D-F:** Micro-CT analysis of distal femur trabecular bone volume fraction (BV/TV) in young ($n \geq 11/\text{gp}$) and adult ($n = 8/\text{gp}$) mice. Representative reconstructed micro-CT cross-sectional images of distal femur from (**D**) young and (**F**) adult mice. Representative images were captured in the distal femur, extending 0.5 mm proximally from where analysis was initiated. **E:** distal femur trabecular BV/TV. * $p < 0.001$: vs. +/+ VEH; ** $p < 0.05$: vs. +/+ VEH; *** $p < 0.001$: vs. -/- VEH; + $p < 0.01$: vs. +/+ VEH; ++ $p < 0.01$: vs. +/+ VEH; +++ $p < 0.05$: vs. -/- VEH. For comparison of +/+ PTH vs. -/- PTH samples, values were expressed as treatment over control prior to statistical analysis. Data is expressed as mean \pm SEM.

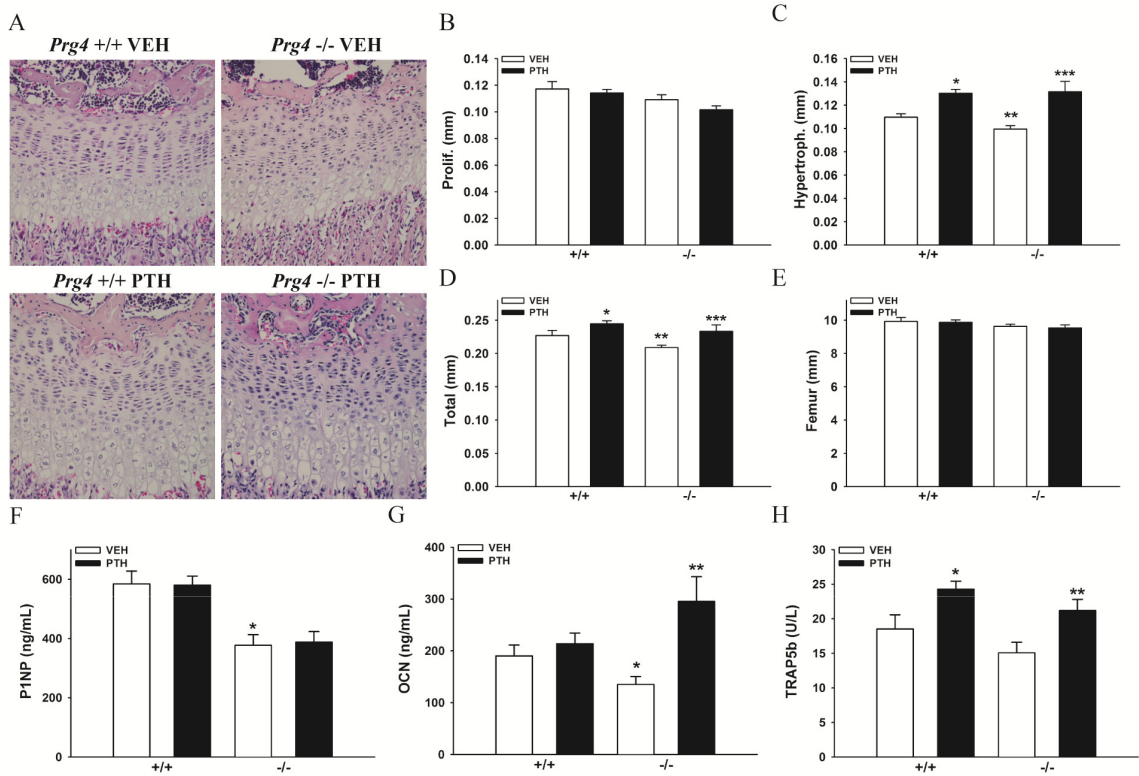


Figure 3.3. Tibial growth plate, femur length, and bone turnover serum biochemical analysis in young mice. **A-H:** Four day old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 17 days (“young” mice). **A-D:** Tibial growth plate morphology and height were evaluated in H&E stained proximal tibia sections from young *Prg4* -/- vs. +/+ mice ($n \geq 11$ /gp). **A:** Representative images (40X) of tibial growth plate from young *prg4* -/- vs. +/+ mice. **B:** proliferative zone height. **C:** hypertrophic zone height. * $p < 0.001$: vs. +/+ VEH; ** $p < 0.05$: vs. +/+ VEH; *** $p < 0.01$: vs. -/- VEH. **D:** total growth plate height. * $p < 0.05$: vs. +/+ VEH; ** $p < 0.05$: vs. +/+ VEH; *** $p < 0.05$: vs. -/- VEH. **E:** Femur length measurements performed via reconstructed micro-CT images of young *Prg4* femurs ($n \geq 11$ /gp). **F-H:** Whole blood was collected via cardiac puncture at euthanasia, and serum isolated for biochemical analysis. Serum N-terminal propeptide of type I procollagen (P1NP) and serum osteocalcin (OCN) were analyzed to assess bone formation, and serum tartrate-resistant acid phosphatase 5b (TRAP5b) was assayed to evaluate bone resorption. **F:** serum P1NP ($n \geq 9$ /gp). * $p < 0.01$: vs. +/+ VEH. **G:** serum OCN ($n \geq 9$ /gp). * $p < 0.05$: vs. +/+ VEH; ** $p < 0.01$: vs. -/- VEH. **H:** serum TRAP5b ($n \geq 9$ /gp). * $p < 0.05$: vs. +/+ VEH; ** $p < 0.05$: vs. -/- VEH. Data is expressed as mean \pm SEM.



Figure 3.4. Bone marrow stromal cell (BMSC) *in vitro* osteoblastogenesis assays. **A-C:** Untreated sixteen week *Prg4* mutant (-/-) and wildtype (+/+) mice were sacrificed, femoral and tibial bone marrow was harvested, and bone marrow stromal cells (BMSCs) were isolated for *in vitro* osteoblastogenesis assays. **A:** cell numbers over time in BMSC cultures ($n \geq 6$ /gp). **B:** Representative images of BMSC d14 von Kossa mineralization cultures. **C:** mineralization area per well in BMSC cultures ($n \geq 6$ /gp). *In vitro* assays were carried out at least three times with similar results. Data is expressed as mean \pm SEM.

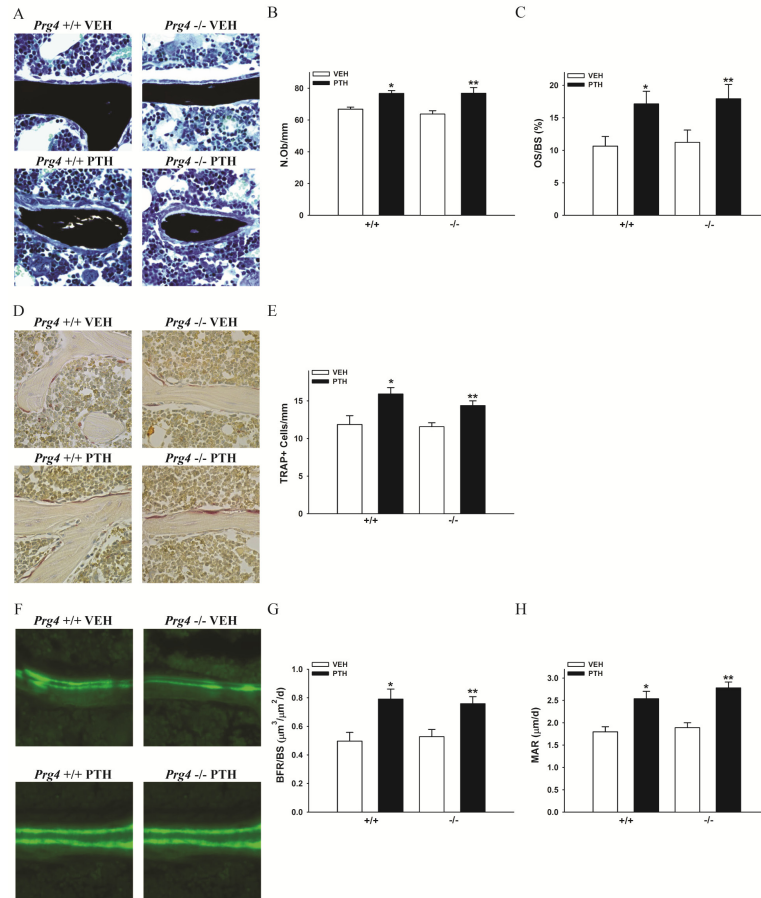


Figure 3.5. Proximal tibia bone cell numbers and activity. **A-H:** Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 $\mu\text{g}/\text{kg}$) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks (“adult” mice). Tibiae were isolated from adult *Prg4* -/- and +/+ mice for histomorphometric analysis of bone cell numbers and activity. **A-C:** Histomorphometric analysis of von Kossa (with tetrachrome counterstain) stained proximal tibia sections was performed to assess osteoblast number per bone perimeter (N.Ob/B.Pm) and osteoid surface (OS/BS) in the secondary spongiosa. **A:** Representative images (40X) of von Kossa stained proximal tibia secondary spongiosa. **B:** osteoblast numbers ($n \geq 10/\text{gp}$). * $p < 0.001$: vs. +/+ VEH; ** $p < 0.01$: vs. -/- VEH. **C:** osteoid surface ($n \geq 10/\text{gp}$). * $p < 0.05$: vs. +/+ VEH; ** $p < 0.05$: vs. -/- VEH. **D** and **E:** TRAP+ cell enumeration was carried out in proximal tibia sections to assess osteoclast numbers per bone perimeter in the secondary spongiosa. **D:** Representative images (40X) of TRAP stained proximal tibia secondary spongiosa. **E:** TRAP+ cell number per bone perimeter ($n \geq 8/\text{gp}$). * $p < 0.05$: vs. +/+ VEH; ** $p < 0.01$: vs. -/- VEH. **F-H:** Dynamic histomorphometric analysis of calcein labeled proximal tibia sections was performed to assess bone formation rates (BFR/BS) and mineral apposition rates (MAR) in the secondary spongiosa. **F:** Representative images (40X) of dual calcein labels in proximal tibia secondary spongiosa. **G:** bone formation rate ($n \geq 8/\text{gp}$). * $p < 0.01$: vs. +/+ VEH; ** $p < 0.01$: vs. -/- VEH. **H:** mineral apposition rate ($n \geq 8/\text{gp}$). * $p < 0.01$: vs. +/+ VEH; ** $p < 0.01$: vs. -/- VEH. Data is expressed as mean \pm SEM.

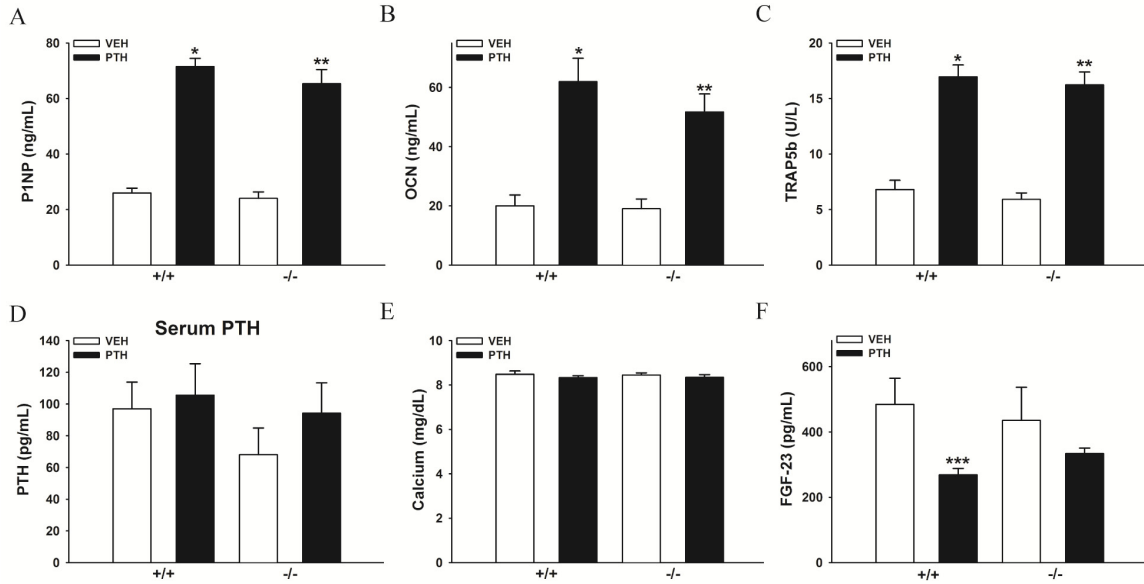


Figure 3.6. Adult *Prg4* mice - serum biochemical analysis of bone turnover and mineral homeostasis. **A-F:** Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks (“adult” mice). Whole blood was collected via cardiac puncture at euthanasia, and serum has isolated for biochemical analysis. **A-C:** Serum N-terminal propeptide of type I procollagen (P1NP) and serum osteocalcin (OCN) were analyzed to assess bone formation, and serum tartrate-resistant acid phosphatase 5b (TRAP5b) was assayed to evaluate bone resorption. Bar graphs represent (A) serum P1NP ($n \geq 9$ /gp), (B) serum OCN ($n \geq 8$ /gp), and (C) serum TRAP5b ($n=10$ /gp). **D-F:** Serum intact parathyroid hormone (1-84) (PTH), serum calcium, and serum fibroblast growth factor-23 (FGF-23) were assayed to assess mineral homeostasis. Bar graphs represent (D) serum PTH ($n \geq 19$ /gp), (E) serum calcium ($n=10$ /gp), (F) and serum FGF-23 ($n=10$ /gp). * $p < 0.001$: vs. +/+ VEH; ** $p < 0.001$: vs. -/- VEH; * $p < 0.05$: vs. +/+ VEH. Data is expressed as mean \pm SEM.

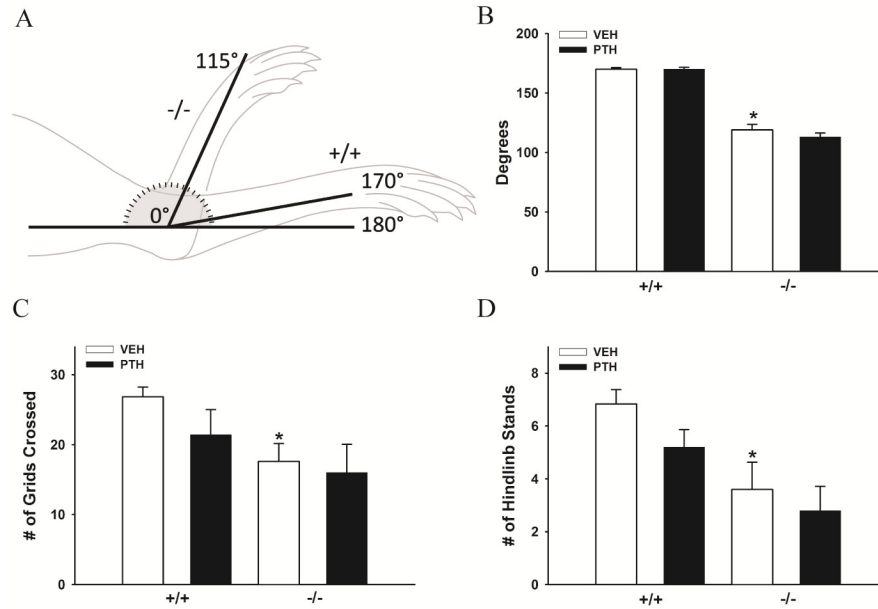


Figure 3.7. Joint range of motion and animal mobility. **A-D**: Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 $\mu\text{g}/\text{kg}$) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks (“adult” mice). **A** and **B**: Maximal hind paw extension was measured to assess joint range of motion. **A**: Tibia was immobilized at 0° and the maximal extension of the hind paw was measured. **B**: maximal hind paw extension ($n \geq 5/\text{gp}$). * $p < 0.001$: vs. +/+ VEH. **C** and **D**: Spontaneous exploratory behavior was assessed to evaluate animal mobility. Mice were placed in a twelve grid chamber for one minute; parameters assessed included number of grids crossed and number of hindlimb stands. **C**: number of grids crossed ($n \geq 5/\text{gp}$). * $p < 0.01$: vs. +/+ VEH. **D**: number of hindlimb stands ($n \geq 5/\text{gp}$). * $p < 0.05$: vs. +/+ VEH. Data is expressed as mean \pm SEM.

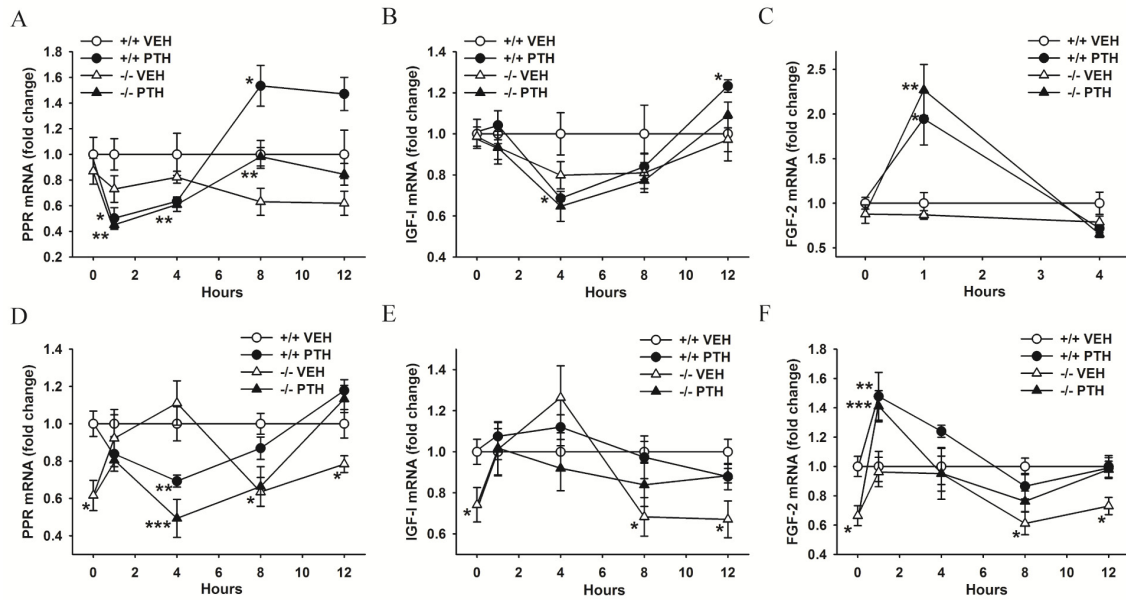


Figure 3.8. PTH regulation of bone marrow and liver gene expression. **A-F:** Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered a single subcutaneous injection of PTH (1-34) (1 μ g/g) or vehicle (VEH) (0.9% NaCl) control, sacrificed 0 (no treatment control), 1, 4, 8, or 12 hours later, and long bone marrow ($n \geq 5$ /gp) and whole liver ($n \geq 5$ /gp) harvested for gene expression analysis. RNA was isolated and quantitative real-time PCR was performed to assess marrow; **(A)** PTH/PTHrP receptor (PPR), **(B)** insulin-like growth factor I (IGF-I), **(C)** basic fibroblast growth factor 2 (FGF-2), and liver; **(D)** PPR, **(E)** IGF-I, **(F)** FGF-2 mRNA expression (standardized to GAPDH levels). Relative quantification of data was determined using the comparative CT method. Line graphs represent PTH effects on mRNA expression over time. **A:** Marrow PPR mRNA; * $p < 0.05$: +/+ PTH vs. +/+ VEH; ** $p < 0.05$: -/- PTH vs. -/- VEH. **B:** Marrow IGF-I mRNA; * $p < 0.05$: +/+ PTH vs. +/+ VEH. **C:** Marrow FGF-2 mRNA; * $p < 0.05$: +/+ PTH vs. +/+ VEH; ** $p < 0.01$: -/- PTH vs. -/- VEH. **D:** Liver PPR mRNA; * $p < 0.05$: -/- VEH vs. +/+ VEH; ** $p < 0.05$: +/+ PTH vs. +/+ VEH; *** $p < 0.01$: -/- PTH vs. -/- VEH. **E:** Liver IGF-I mRNA; * $p < 0.05$: -/- VEH vs. +/+ VEH. **F:** Liver FGF-2 mRNA; * $p < 0.05$: -/- VEH vs. +/+ VEH; ** $p < 0.05$: +/+ PTH vs. +/+ VEH; *** $p < 0.05$: -/- PTH vs. -/- VEH. Data is expressed as mean \pm SEM.

Young <i>Prg4</i> mice	+/+ VEH	+/+ PTH	-/- VEH	-/- PTH
Trabecular bone				
BV/TV (%)	8.50 ± 0.57	18.8 ± 1.64***	7.00 ± 0.39*	17.5 ± 1.83+++
Tb.Th (µm)	27.7 ± 0.64	36.5 ± 1.21***	26.6 ± 0.32	33.3 ± 1.55+++
Tb.Sp (µm)	309 ± 18.2	167 ± 11.5***	365 ± 19.0*	177 ± 20.0+++
Tb.N (mm ⁻¹)	3.04 ± 0.15	5.07 ± 0.29***	2.62 ± 0.13*	5.11 ± 0.38+++
Tb.BMD (mg/cc)	134 ± 3.54	191 ± 9.19***	123 ± 3.38*	187 ± 9.65+++
Cortical bone				
Tt.Ar (mm ²)	1.37 ± 0.07	1.32 ± 0.04	1.35 ± 0.04	1.26 ± 0.04
Ct.Ar (mm ²)	0.37 ± 0.03	0.33 ± 0.02	0.34 ± 0.01	0.31 ± 0.02
Ct.Ar/Tt.Ar (%)	26.6 ± 0.82	24.5 ± 1.02	25.2 ± 0.60	24.2 ± 0.98
Ct.Th (µm)	96.1 ± 5.43	86.2 ± 5.09	89.5 ± 3.05	83.3 ± 4.54
Ma.Ar (mm ²)	1.00 ± 0.04	0.99 ± 0.02	1.01 ± 0.03	0.96 ± 0.02
Ec.Pm (mm)	3.61 ± 0.08	3.61 ± 0.04	3.62 ± 0.05	3.53 ± 0.04
Ps.Pm (mm)	4.20 ± 0.11	4.14 ± 0.07	4.17 ± 0.06	4.05 ± 0.06
BMD (mg/cc)	235 ± 8.52	207 ± 10.5	219 ± 7.42	208 ± 9.01
Adult <i>Prg4</i> mice				
Trabecular bone				
BV/TV (%)	10.7 ± 0.87	20.3 ± 2.40** (89%)	6.79 ± 0.57**	9.78 ± 0.90 ⁺ (44%)
Tb.Th (µm)	32.5 ± 0.61	45.0 ± 2.99** (39%)	29.9 ± 1.70	33.9 ± 1.50 [#] (13%)
Tb.Sp (µm)	283 ± 21.9	188 ± 18.7** (-34%)	424 ± 29.9**	320 ± 24.5 ⁺ (-25%)
Tb.N (mm ⁻¹)	3.28 ± 0.23	4.49 ± 0.36* (37%)	2.26 ± 0.13**	2.88 ± 0.23 ⁺ (28%)
Tb.BMD (mg/cc)	197 ± 11.8	278 ± 19.3*** (41%)	168 ± 9.42*	184 ± 9.85 ^{+,#} (16%)
Cortical bone				
Tt.Ar (mm ²)	1.74 ± 0.03	1.94 ± 0.05** (12%)	1.80 ± 0.03	1.88 ± 0.04 [#] (4.1%)
Ct.Ar (mm ²)	0.88 ± 0.01	1.02 ± 0.02** (17%)	0.83 ± 0.02	0.86 ± 0.02 ^{##} (3.7%)
Ct.Ar/Tt.Ar (%)	50.5 ± 0.66	52.7 ± 0.64* (4.2%)	46.1 ± 0.88**	45.9 ± 0.37 ^{##} (-0.3%)
Ct.Th (µm)	221 ± 3.06	246 ± 3.15** (11%)	202 ± 5.34**	204 ± 3.52 ^{###} (1.3%)
Ma.Ar (mm ²)	0.86 ± 0.02	0.92 ± 0.03 (7.2%)	0.97 ± 0.02**	1.02 ± 0.02 (4.4%)
Ec.Pm (mm)	3.50 ± 0.05	3.63 ± 0.07 (3.6%)	3.61 ± 0.03	3.69 ± 0.04 (2.1%)
Ps.Pm (mm)	4.89 ± 0.05	5.15 ± 0.09* (5.4%)	4.91 ± 0.06	4.97 ± 0.05 (1.3%)
BMD (mg/cc)	511 ± 9.46	562 ± 14.2** (10%)	483 ± 15.0	491 ± 6.14 [#] (1.6%)

Table 3.1. Femur micro-CT analysis. Trabecular bone structural parameters: BV/TV (bone volume); Tb.Th (trabecular thickness); Tb.Sp (trabecular separation); Tb.N (trabecular number); Tb.BMD (trabecular bone mineral density). Cortical bone structural parameters: Tt.Ar (total area); Ct.Ar (cortical area); Ct.Ar/Tt.Ar (cortical area fraction); Ct.Th (cortical thickness); Ma.Ar (marrow area); Ec.Pm (endocortical perimeter); Ps.Pm (periosteal perimeter); BMD (bone mineral density). N-values: young *prg4* mice (n≥11/gp) and adult *Prg4* mice (n=8/gp). * p<0.05 vs. +/+ VEH; ** p<0.01 vs. +/+ VEH; *** p<0.001 vs. +/+ VEH; ⁺p<0.05 vs. -/- VEH; ⁺⁺p<0.01 vs. -/- VEH; ⁺⁺⁺p<0.001 vs. -/- VEH; [#]p<0.05 vs. +/+ PTH; ^{##}p<0.01 vs. +/+ PTH, ^{###}p<0.001 vs. +/+ PTH. For comparison of +/+ PTH vs. -/- PTH samples, values were expressed as treatment over control prior to statistical analysis. Percentage increase over control analysis is presented in parenthesis. Data is expressed as mean ± SEM.

Adult <i>Prg4</i> mice	+/+ VEH	+/+ PTH	-/- VEH	-/- PTH
<i>Marrow mRNA</i>				
PPR	1.00 ± 0.19	0.84 ± 0.06	0.77 ± 0.08	0.64 ± 0.05
IGF-I	1.00 ± 0.03	0.80 ± 0.05*	0.89 ± 0.09	0.68 ± 0.04
FGF-2	1.00 ± 0.08	1.01 ± 0.05	0.68 ± 0.06*	0.91 ± 0.08⁺
<i>Liver mRNA</i>				
PPR	1.00 ± 0.08	0.98 ± 0.14	0.68 ± 0.06*	0.86 ± 0.09
IGF-I	1.00 ± 0.14	1.02 ± 0.13	0.59 ± 0.06*	1.03 ± 0.07⁺⁺
FGF-2	1.00 ± 0.14	1.17 ± 0.16	0.63 ± 0.04*	1.21 ± 0.11⁺⁺

Table 3.2. Bone marrow and liver gene expression. Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 µg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks (“adult” mice). Bone marrow (n=4-5/gp) and whole liver (n=5-6/gp) were harvested for gene expression analysis. RNA was isolated and quantitative real-time PCR was performed to assess PTH/PTHrP receptor (PPR), insulin-like growth factor I (IGF-I), and basic fibroblast growth factor 2 (FGF-2) mRNA expression (standardized to GAPDH levels). Relative quantification of data generated was carried out using the comparative CT method. *p<0.05 vs. +/+ VEH; ⁺p<0.05 vs. -/- VEH, ⁺⁺p<0.01 vs. -/- VEH. Data is expressed as mean ± SEM.

Adult <i>Prg4</i> mice	+/+ VEH	+/+ PTH	-/- VEH	-/- PTH
<i>Serum protein</i>				
IGF-I (ng/mL)	603.19 ± 28.78	589.78 ± 7.38	538.29 ± 32.52	554.81 ± 24.79
FGF-2 (pg/mL)	4.00 ± 0.76	3.82 ± 1.13	1.07 ± 0.30**	5.32 ± 1.12⁺⁺

Table 3.3. Serum biochemical analysis – IGF-I and FGF-2. Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 µg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks (“adult” mice). Whole blood was collected via cardiac puncture at euthanasia, and serum has isolated for biochemical analysis of insulin-like growth factor I (IGF-I) (n=4-5/gp) and basic fibroblast growth factor 2 (FGF-2) (n=5-6/gp). ** p<0.01: vs. +/+ VEH; ⁺⁺p<0.01: vs. -/- VEH. Data is expressed as mean ± SEM.

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CHAPTER 4

THE IMPACT OF PROTEOGLYCAN 4 AND PARATHYROID HORMONE ON ARTICULAR CARTILAGE DEGENERATION

Abstract

Proteoglycan 4 (*Prg4*), a prominent boundary lubricating factor, protects synovial joints from arthropathic changes by mechanisms that are incompletely understood.

Parathyroid hormone (PTH), known for its anabolic actions in bone, has been reported to protect articular cartilage from degeneration in arthropathic joints. To investigate the effect of proteoglycan 4 and the impact of PTH on articular cartilage in degenerating joints, 16 week old male *Prg4* mutant and wildtype mice were treated with intermittent PTH (1-34) or vehicle control subcutaneous injection daily from 16 until 22 weeks of age. Analyses included histology of the knee joint, micro-CT imaging of the distal femur, and serum biochemical analysis of type II collagen fragments (CTX-II).

Results: Compared to wildtype littermates, *Prg4* mutant mice had a loss of superficial zone chondrocytes, an acellular layer of material lining the surfaces of the articular cartilage and menisci, increased articular cartilage degradation, increased serum CTX-II concentrations, decreased articular chondrocyte apoptosis, increased synovium SDF-1 expression, and irregularly contoured subchondral bone. *Prg4* mutant mice that received intermittent PTH administration developed a secondary deposit overlaying the acellular

layer of material lining the joint surfaces. The finding that *Prg4* mutant mice have supra-physiologic SDF-1 levels in the joint introduces a strong candidate non-mechanical mechanism by which PRG4 protects articular cartilage. Increased joint SDF-1 levels likely contribute to the loss of superficial zone chondrocytes and increased cartilage degradation in *Prg4* mutant mice. Intermittent PTH-treatment did not lessen signs of articular cartilage degeneration in *Prg4* mutant mice.

Introduction

Proteoglycan 4 (*Prg4*) is essential for protecting joints. The *Prg4* protein products, lubricin and superficial zone protein (SZP), are secreted glycoproteins which are expressed in synovial joints by synoviocytes and superficial zone articular chondrocytes (1;2). Characterization of the articular joints in *Prg4* mutant (-/-) mice demonstrated that absence of proteoglycan 4 causes hyperplasia of synovial intimal cells, disappearance of superficial zone chondrocytes, abnormal protein deposition on cartilage surfaces, articular cartilage degradation, and ultimately precocious joint failure (1;3). While it has been shown that proteoglycan 4 prevents synoviocyte hyperplasia by restricting adhesion dependent synovial intimal cell proliferation *ex vivo* (1), the mechanism(s) by which proteoglycan 4 regulates synoviocyte proliferation and protects articular cartilage remain unclear.

Intermittent parathyroid hormone (PTH) (1-34) injection, currently the only FDA approved anabolic therapy for osteoporosis, has recently been reported to inhibit the progression of articular cartilage degeneration (4;5). In bone PTH exerts its anabolic

effects by signaling at stromal/osteoblastic cells, the predominant PTH/PTH-related protein (PTHrP) receptor (PPR) expressing cells (6). While the PPR is expressed at highest levels in bone and kidney, PPR is also expressed in cartilage. During skeletal development the PPR is expressed in growth plate prehypertrophic zone chondrocytes (7;8), contributing to a signaling feedback loop involving indian hedgehog which regulates endochondral bone formation (9-11). While the PPR is also expressed in articular chondrocytes (5;12), the role of PPR signaling in articular cartilage is unclear.

PRG4 loss-of-function mutations in humans result in an autosomal recessive disorder, camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, which is characterized by precocious joint failure (13). In addition to CACP syndrome, *PRG4* is reduced in non-inflammatory and inflammatory mediated degenerative joint diseases, including osteoarthritis (OA) (14;15) and rheumatoid arthritis (RA) (16), which suggests that decreased *PRG4* levels contribute to degenerative joint disease.

Based on the association between reduced *PRG4* levels and degenerative joint disease (14-16), and reports that PTH protects articular cartilage from degeneration in arthropathic joints, we hypothesized that proteoglycan 4 supports PTH actions in the joints. *Prg4*^{-/-} mice (1) were studied to identify proteoglycan 4 functions regulating articular cartilage protection *in vivo*. We also wanted to determine whether intermittent PTH administration could inhibit articular cartilage degeneration in arthropathic joints deficient in proteoglycan 4.

Materials and methods

Breeding of *Prg4* mutant mice, and administration of PTH

The generation of a *Prg4* mutant allele has been previously described (1). We backcrossed the allele onto a C57BL6 genetic background. *Prg4* +/- mice were crossed and the male homozygous null (-/-) and wildtype (+/+) offspring were retained. Beginning at 16 weeks of age, *Prg4* -/- and +/+ littermates were given daily subcutaneous injections of either recombinant human PTH (1-34) (50 µg/kg) (Bachem, Torrance, CA) or vehicle (0.9% NaCl) for 6 weeks. Twenty-four hours following the final injection, the mice were sacrificed via decapitation and their tissues were harvested for analyses. All animal studies were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA), and animals were maintained in accordance with approved UCUCA research protocols.

Histopathology

Hindlimbs were fixed in 10% phosphate-buffered formalin for 48 hours at 4°C, and then decalcified in 14% EDTA pH 7.2 for 14 days at room temperature. Knee joints were embedded in paraffin, and 5 µm serial sections cut and stained. Serial sagittal knee sections were collected, beginning laterally and moving medially across the knee joint. Hematoxylin & eosin (H&E) and Safranin O-fast green stains were performed. H&E stained sections were assessed for histopathology. Safranin O-fast green stained sections were evaluated for articular cartilage degradation. The Osteoarthritis Research Society International (OARSI) osteoarthritis cartilage histopathology assessment system (17) was adopted to score the severity and extent of cartilage degradation in the tibial plateau (18).

Serum biochemical analysis

Whole blood was collected via cardiac puncture at euthanasia, coagulated at room temperature for 30 min, centrifuged, serum isolated, and stored at -80°C. Serum Pre-Clinical CartiLaps ELISA (Immunodiagnostic Systems, Fountain Hills, AZ) was carried out in duplicate, per manufacturer's instructions, to assess degradation products of C-terminal telopeptides of type II collagen (CTX-II).

Micro-CT

Femurs were fixed in 10% phosphate buffered formalin for 48 h at 4°C, then stored in 70% ethanol. Femurs were scanned in water at an 18- μ m isotropic voxel resolution via eXplore Locus SP (GE Healthcare Pre-Clinical Imaging, London, ON, Canada), and calibrated three-dimensional images were reconstructed. Distal femurs were assessed via GE Medical Systems MicroView v2.2 software (GE Healthcare Pre-Clinical Imaging). Perpendicular to a line drawn through the apices of the lateral edges of the patellar groove, the depth of the patellar groove was measured. A fixed threshold of 1200 Hounsfield Units for subchondral bone was used to discriminate mineralized tissue (19).

TUNEL immunohistochemistry (IHC)

Immunohistochemical analysis of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was carried out in sagittal knee sections to assess chondrocyte apoptosis in articular cartilage. The region of interest was the full thickness cartilage of the tibial plateau, between the anterior and posterior menisci. Sections were deparaffinized with mixed xylenes and re-hydrated in graded ethanols. Exposed ends of DNA fragments, induced by apoptotic signals, were bound by Klenow enzyme and

subsequently detected following the protocol of the FragEL™ DNA Fragmentation Detection Kit (EMD Chemicals, Gibbstown, NJ).

SDF-1 immunofluorescence (IF)

Stromal cell-derived factor-1 (SDF-1) immunofluorescence (IF) was carried out in sagittal knee sections to assess SDF-1 expression in the synovium. Samples were deparaffinized with mixed xylenes, re-hydrated in graded ethanols, then briefly washed with PBT (PBS plus 0.2% Triton X-100) and blocked for 30 minutes with Image-iT FX Signal Enhancer (Invitrogen, Carlsbad, CO). SDF-1 IF was performed via Zenon Alexa Fluor 488 Rabbit Labeling Kit (Invitrogen) and SDF-1 alpha rabbit polyclonal antibody (Catalogue number: ab25117) (ABCAM, Cambridge, MA). After overnight incubation at 4° with 1ug/100ul dilution of fluorescence-labeled SDF-1 alpha rabbit polyclonal antibody, sections were washed with PBT, mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), and cover slipped. Images were acquired with Olympus FV-500 confocal microscope. Image analysis of SDF-1 IF in the synovium was performed using Image Pro Plus 5.1 Software (Media Cybernetics, Silver Spring, MD). Two-three representative images (20X) of the synovium were acquired in the anterior aspect of the knee joint, and IF image settings were consistent for all *Prg4*^{-/-} and *Prg4*^{+/+} tissue sections.

Statistical analyses

Unpaired *t* tests were performed using GraphPad InStat Software (GraphPad Software, San Diego, CA). Statistical significance was noted at $p < 0.05$. Data are presented as mean \pm standard error of mean (SEM).

Results

Histological abnormalities in the cartilage and meniscus of *Prg4*^{-/-} mice

Histopathologic assessment of hematoxylin and eosin (H&E) stained sagittal knee sections demonstrated that vehicle- and PTH-treated *Prg4*^{-/-} mice had disappearance of superficial zone chondrocytes, articular cartilage degradation, and an acellular layer of material lining the surfaces of the articular cartilage and menisci (**Figure 4.1**). Rhee et al. (1) speculated that the acellular layer of material lining the *Prg4*^{-/-} joint surfaces is composed of adsorbed proteins from the synovial fluid. Interestingly, the formation of a secondary deposit overlaying the acellular layer of material lining the surfaces of the articular cartilage and posterior meniscus was detected in 5 of 10 PTH-treated *Prg4*^{-/-} mice. None (0 of 10) of the vehicle-treated *Prg4*^{-/-} mice demonstrated the secondary deposits. PTH-treatment had no observed effects on the joints of *Prg4*^{+/+} mice (**Figure 4.1**).

Irregular subchondral bone in *Prg4*^{-/-} mice

Reconstructed micro-CT images of the distal femur were assessed to confirm the expected bone forming anabolic effects of PTH in *Prg4*^{-/-} and *+/+* mice. Micro-CT sagittal section images of the distal femur visually demonstrated that PTH therapy increased metaphyseal trabecular and cortical bone in *Prg4*^{-/-} and *+/+* mice (**Figure 4.2A**). While a side view (**Figure 4.2B**) and posterior view (**Figure 4.2C**) demonstrated no obvious morphological differences, the anterior view of the distal femur (**Figure 4.2D**) revealed abnormalities in the surface contour of the subchondral bone in *Prg4*^{-/-} mice. Specifically, the subchondral bone of the patellar groove had irregular contours

characterized by a medial clefting with elevated lateral edges (**Figure 4.2D**).

Perpendicular to a line drawn through the apices of the lateral edges, the depth of the patellar groove was significantly greater in *Prg4* *-/-* and *+/+* mice (**Figure 4.2D,E**).

Findings that *Prg4* mRNA is expressed in bone (20) and the *Prg4* *-/-* mice and humans with CACP have periarticular osteopenia (1;13) suggest that lack of proteoglycan 4 affects the morphology of subchondral bone.

Articular cartilage degradation in *Prg4* *-/-* mice

Histopathologic assessment of Safranin O-fast green stained sagittal knee sections revealed that *Prg4* *-/-* mice had articular cartilage surfaces characterized by fibrillations, delaminations, fissures and mid-zone excavations (**Figure 4.3A**). Histologic scoring indicated that the severity and extent of articular cartilage degradation was greater in *Prg4* *-/-* vs. *+/+* mice, and that the severity was not altered by PTH-treatment (**Figure 4.3B**). Serum C-terminal telopeptides of type II collagen (CTX-II) were assayed as a marker for articular cartilage degradation. Serum CTX-II levels were increased in *Prg4* *-/-* vs. *+/+* mice, and were not affected by PTH-treatment (**Figure 4.3C**).

Decreased articular chondrocyte apoptosis in *Prg4* *-/-* mice

TUNEL labeling of sagittal knee sections was performed to evaluate the effect of proteoglycan 4 and the impact of PTH-treatment on articular chondrocyte apoptosis. The frequency of TUNEL+ chondrocytes was significantly lower in *Prg4* *-/-* vs. *+/+* mice. There was no effect of PTH on the frequency of TUNEL+ chondrocytes in the articular cartilage of either *Prg4* *-/-* or *+/+* mice (**Figure 4.4A,B**). There was no difference in the total number of chondrocytes per cartilage area in *Prg4* *-/-* vs. *+/+* mice, and PTH-treatment did not alter total number of chondrocytes per cartilage area (**Figure 4.4C**).

Increased SDF-1 expression in the synovium of *Prg4* ^{-/-} mice

The expression of stromal-cell derived factor-1 (SDF-1), a cytokine that plays a multifaceted role in degenerative joint diseases, was assessed in the synovium since PTH has been shown to regulate SDF-1 in diverse tissues, including bone (21) and heart (22). Immunofluorescence (IF) labeling of sagittal knee sections revealed significantly increased SDF-1 in the synovium of *Prg4* ^{-/-} vs. ^{+/+} mice (**Figure 4.5A,B**). There was no difference in SDF-1 expression in the synovium of vehicle- vs. PTH-treated mice. While the *Prg4* ^{+/+} synovium was characterized by low to moderate intensity SDF-1 expression limited to the lining synoviocytes, the *Prg4* ^{-/-} synovium had intense SDF-1 expression in the hyperplastic lining. In addition, perivascular endothelial cells expressed SDF-1 in the synovium of *Prg4* ^{-/-} mice (**Figure 4.5A**).

Discussion

Reduced PRG4 levels have been associated with osteoarthritis (OA) and rheumatoid arthritis (RA), the two most common degenerative joint conditions. The finding that PRG4 levels are decreased in the synovial fluid of patients following anterior cruciate ligament injury, a significant risk factor for OA (23), suggests that reducing the amount of PRG4 places joints at risk for OA. Animal models are also consistent with this hypothesis since joint injury models have down-regulated *Prg4* expression in the superficial zone cartilage and decreased proteoglycan 4 abundance in synovial fluid (14;15). Gene expression analysis of synovial tissue harvested from RA patients reveals heterogeneity in *PRG4* levels, distinguishing high and low *PRG4* expressing groups (16).

Low *PRG4* expression is associated with more aggressive stages of RA, which suggests that decreased PRG4 levels may contribute to the progression of RA (16). These reports of decreased PRG4 levels in arthropathic joints, coupled with studies demonstrating that exogenous lubricin administration inhibits the onset and progression of OA in rats (24;25) suggest that PRG4 plays a regulatory role in the pathogenesis of degenerative joint disease.

While systemic PTH (1-34) administration decelerated articular cartilage degeneration in a murine surgically induced OA model (4), and intra-articular PTH (1-34) injection inhibited the progression of articular cartilage degeneration in a rat chemically induced OA model (5), the mechanisms mediating such protective effects in the joints are unclear. Similar to PTHrP actions in the growth plate which inhibit chondrocyte maturation towards hypertrophy (9-11), *in vitro* PTH/PTHrP administration to articular chondrocytes, cultured under differentiation conditions, has been reported to suppress chondrocyte hypertrophy (26) and reverse terminal differentiation (5). Based on studies reporting that articular chondrocyte maturation is accelerated in OA joints, ultimately resulting in mineralization and apoptosis (27;28), the finding that intra-articular PTH administration significantly suppresses chondrocyte apoptosis in a rat OA model (5) suggests that PTH protects articular cartilage from arthropathic changes by restricting chondrocyte maturation.

The present study tested whether intermittent PTH administration would protect joints in *Prg4* mutant mice. Treatment began at 16 weeks in mice, which is an age at which *Prg4* mutant mice have an intermediate degree of joint failure, as indicated by a significant loss of cartilage structure, stiffness, and frictional properties (1;3). The

intermediate stage was evaluated since this is the period when the majority of arthropathic patients become symptomatic and present for clinical care. While the intra-articular injection method delivers a controlled local dose to a targeted joint, PTH was administered via subcutaneous injection to avoid the risk of iatrogenic trauma to the joints.

Surprisingly, the only observed PTH effect in the joints of *Prg4* mutant mice was that PTH induced the formation of a secondary deposit overlaying the acellular layer of material lining the surfaces of the articular cartilage and posterior meniscus in half of the PTH-treated mutant animals. There were no other observed differences in the joints of PTH-treated *Prg4* mutant mice, and although the nature of this deposit is unknown at present, its formation suggests that PTH signaling was functional within the joints. Similar to investigators who reported that PTH did not affect articular chondrocytes in the knee joints of healthy rats (5), systemic PTH administration had no impact on the joints of *Prg4* wildtype mice.

The minimal impact of PTH in the arthropathic joints of *Prg4* mutant mice, combined with previous findings that *Prg4* is a PTH-responsive gene (29), suggests that proteoglycan 4 may be required for PTH to exert chondroprotective actions. Taking into consideration a proposed hypothesis that PTH protects articular cartilage from arthropathic changes by restricting chondrocyte maturation (5), PTH may not have altered articular cartilage degeneration in *Prg4* mutant mice due the anti-apoptotic nature of the *Prg4* mutant chondrocytes. However, an alternative explanation would be that damage caused by the complete absence of proteoglycan 4 cannot be modified by the actions of PTH.

The irregularly contoured subchondral bone surface of the patellar groove in *Prg4* mutant mice demonstrates that *Prg4* loss-of-function mutation-induced changes in the joints are not limited to the synovium and cartilage. Of interest, prior work has suggested that changes in the shape and contour of the subchondral bone adversely impact the mechanical loading properties of the adjacent articular cartilage (30;31), which has been speculated to induce pathologic changes in cartilage structure and integrity (32). Regardless of whether changes in subchondral bone precede or are secondary to alterations in articular cartilage, the irregularly contoured subchondral bone likely contributes to the disruption of the articular cartilage in *Prg4* mutant mice.

The increased SDF-1 expression in the synovium of *Prg4* mutant mice is a notable discovery since there are currently no known non-mechanical mechanisms by which proteoglycan 4 protects articular cartilage. While it has been speculated that changes in the articular cartilage of *Prg4* mutant mice are caused by friction induced changes secondary to the lack of boundary lubrication (normally provided by lubricin) (1;33), recent work suggest additional hypotheses. Coles et al. (3) demonstrated that *Prg4* mutant mice have increased articular cartilage surface roughness by 2 weeks of age and increased articular cartilage degeneration by 4 weeks of age, yet the coefficient of friction is not increased in the joints of *Prg4* mutant mice until 16 weeks of age. These data indicate that articular cartilage degeneration in *Prg4* mutant mice may not be secondary to mechanical alterations suggesting non-mechanical mechanisms where by proteoglycan 4 protects articular cartilage.

The supra-physiologic levels of SDF-1 observed in the joints of *Prg4* mutant mice likely mediate non-mechanical induced alterations in the articular cartilage. SDF-1, a

chemokine best known for its physiologic roles regulating the homing and mobilization of hematopoietic cells in the bone marrow (34;35), plays a multifaceted role in joint disease. Within articular joints SDF-1 is expressed and secreted by synoviocytes, and the SDF-1 receptor, CXCR4, is expressed by articular chondrocytes (36;37). While there is no known physiologic role for SDF-1/CXCR4 paracrine signaling in articular joints, SDF-1 levels are increased in the joints of patients afflicted by OA and RA (37;38). Recent work implicates supra-physiologic joint SDF-1 levels in articular cartilage degradation (37;38), chondrocyte cell death (39), synovium angiogenesis (36), and T-cell recruitment to the synovium (40;41).

In light of *in vitro* articular chondrocyte studies demonstrating that supra-physiologic SDF-1 levels stimulate the secretion of matrix metalloproteinase-3 (MMP-3) (37), MMP-9, and MMP-13 (38), we speculate that increased SDF-1 levels in the joints of *Prg4* mutant mice stimulate articular chondrocytes to release MMPs which mediate the accelerated degradation of articular cartilage. Since MMP mediated degradation of articular cartilage is required for the release of collagen type-II fragments into the circulation, the increased serum CTX-II levels observed provides evidence that MMP activity is up-regulated in the joints of *Prg4* mutant mice.

Taking into account prior *in vitro* work reporting that supra-physiologic SDF-1 levels induce chondrocyte cell death via a necrosis-dependent mechanism (39), increased SDF-1 levels in the joints of *Prg4* mutant mice may play a role in the loss of superficial zone articular chondrocytes. Increased SDF-1 levels in the joints of *Prg4* mutant mice were not associated with an increase in articular chondrocyte apoptosis, which is consistent with prior *in vitro* work reporting that supra-physiologic SDF-1 levels do not

induce chondrocyte cell death via an apoptosis dependent mechanism (39). The unexpected finding that apoptosis was significantly decreased in articular chondrocytes in *Prg4* mutant mice suggests that *Prg4* mutant articular chondrocytes may have cell autonomous alterations protecting against apoptosis. Alternatively, based on studies reporting that SDF-1 has anti-apoptotic effects on mesenchymal stem cells (42), hematopoietic progenitor cells (43), and pancreatic beta cells (44), increased SDF-1 levels may inhibit articular chondrocyte apoptosis in the joints of *Prg4* mutant mice.

The intense SDF-1 expression in the hyperplastic lining of the *Prg4* mutant synovium is consistent with RA synovial tissue SDF-1 immunolocalization studies which have reported increased SDF-1 expression of the hyperplastic lining that extends to the extracellular matrix and the perivascular areas of the sublining (36;45). Studies demonstrating that inflammatory cytokines do not impact synoviocyte SDF-1 expression (36;46) and that isolated RA synoviocytes constitutively express SDF-1 at physiologic levels (36;46) suggest that SDF-1 levels are increased in RA synovium due to synoviocyte hyperplasia. Based on studies which imply that the increased SDF-1 levels in RA synovium are due to synovial hyperplasia, the similarities in the intensity and distribution of increased SDF-1 expression in *Prg4* mutant hyperplastic synovium and RA hyperplastic synovium suggest that increased SDF-1 levels in the *Prg4* mutant synovium are secondary to synovial hyperplasia. Therefore, we speculate that proteoglycan 4 restricts SDF-1 expression in the synovium via proteoglycan 4 actions inhibiting synoviocyte hyperplasia.

This investigation of degenerating joints in adult *Prg4* mutant mice uncovered novel candidate mechanisms by which proteoglycan 4 protects articular cartilage. While

it is unclear whether the irregularly contoured subchondral bone in *Prg4* mutant mice is due to proteoglycan 4 actions supporting skeletogenesis or protecting articular cartilage, the altered subchondral bone morphology likely contributes to the disruption of the articular cartilage in mutant mice. The increased joint SDF-1 levels, which appear secondary to the synovial hyperplasia, suggest a non-mechanical mechanism by which proteoglycan 4 protects articular cartilage. In summary, proteoglycan 4 is a dynamic factor that protects articular cartilage by diverse mechanical and non-mechanical mechanisms.

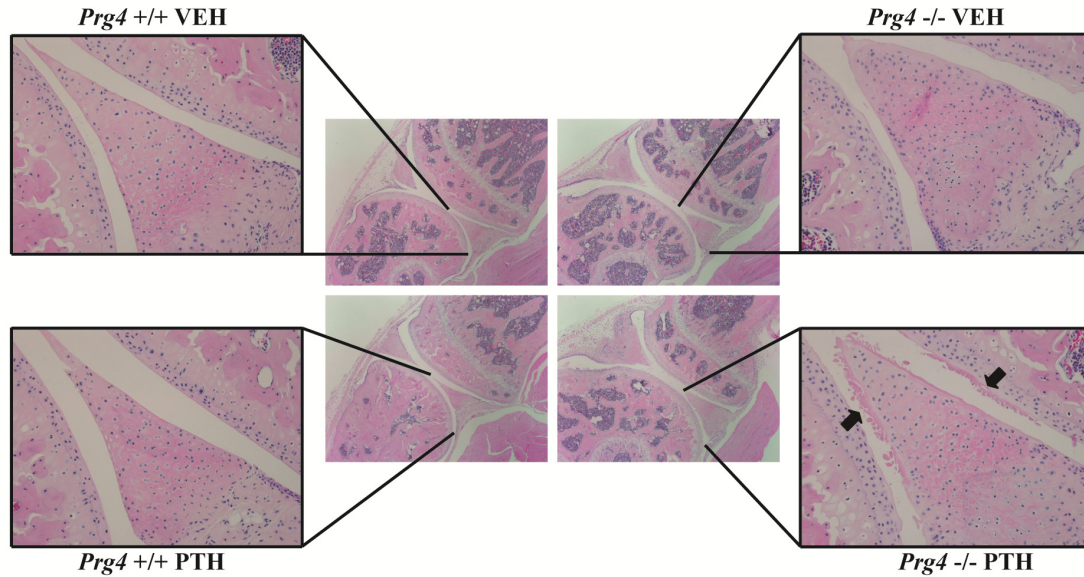


Figure 4.1. Knee joint histopathology. Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 $\mu\text{g}/\text{kg}$) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from 16-22 weeks. Hindlimbs were isolated for histopathologic analysis of the knee joint. Representative images of hematoxylin & eosin (H&E) stained sagittal knee sections (4X), with (inset) 20X image of the posterior meniscus (**black arrows** indicate the PTH-induced secondary deposit) (n=10/gp).

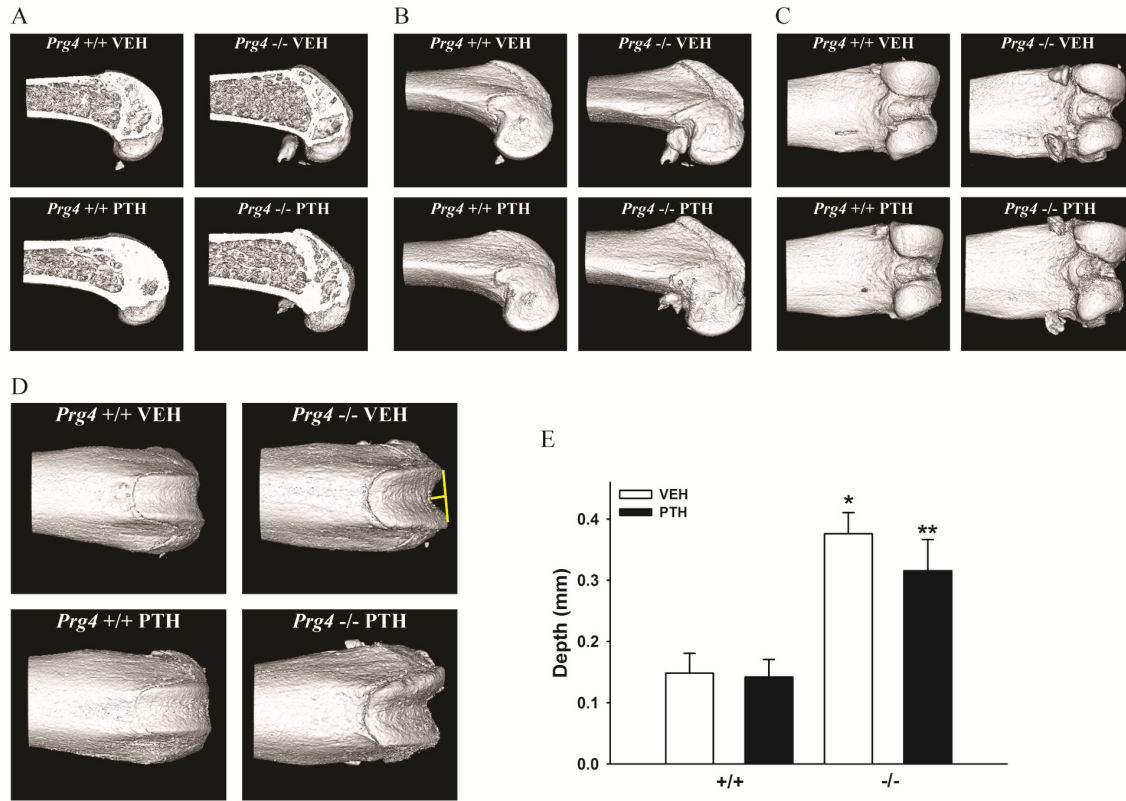


Figure 4.2. Subchondral bone morphology. **A-E:** Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from 16-22 weeks. Representative reconstructed micro-CT images of the distal femur; **(A)** sagittal section view, **(B)** side view, **(C)** posterior view, **(D)** anterior view (**yellow line** indicates depth of the patellar groove). **E:** Bar graph represents patellar groove depth (n=7-8/gp). * p<0.001 vs. +/+ VEH; ** p<0.01 vs. +/+ PTH. Data is expressed as mean \pm SEM.

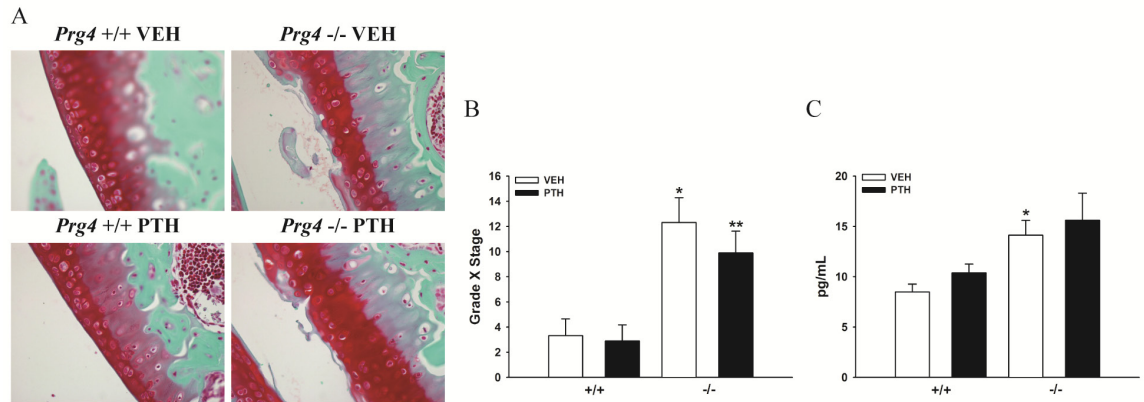


Figure 4.3. Articular cartilage degradation. **A-C:** Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks, from 16-22 weeks. Safranin O-fast green stained sagittal knee sections were assessed for articular cartilage degradation. **A:** Representative images of Safranin O-fast green stained articular cartilage (40X). **B:** The Osteoarthritis Research Society International (OARSI) osteoarthritis cartilage histopathology assessment system was applied to score the severity and extent of articular cartilage degradation in the tibial plateau. Bar graph represents the OARSI score for the articular cartilage in the tibial plateau (n=8-10/gp). * $p < 0.01$ vs. +/+ VEH; ** $p < 0.01$ vs. +/+ PTH. Data is expressed as mean \pm SEM. **C:** Whole blood was collected via cardiac puncture at euthanasia, and serum was isolated for biochemical analysis of C-terminal telopeptides of type II collagen (CTX-II), a marker for type II collagen degradation. Bar graph represents serum CTX-II levels (n=8-10/gp). * $p < 0.01$ vs. +/+ VEH; ** $p = 0.08$ vs. +/+ PTH. Data is expressed as mean \pm SEM.

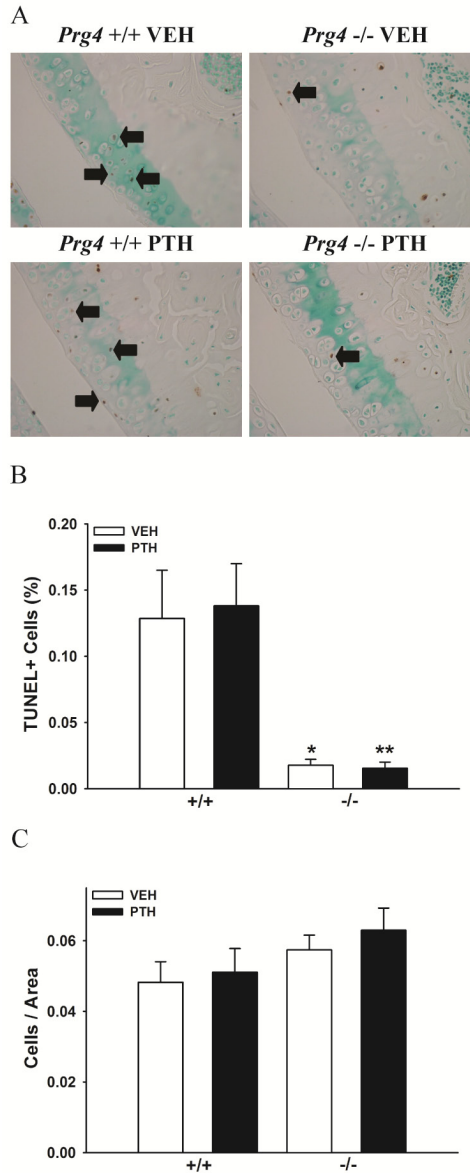


Figure 4.4. Chondrocyte apoptosis. **A-C:** Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks, from 16-22 weeks. Immunohistochemical analysis of TUNEL labeling in sagittal knee sections was carried out to assess articular chondrocyte apoptosis. **A:** Representative images of TUNEL labeled chondrocytes in the articular cartilage (40X) (**black arrows** point to selected TUNEL+ chondrocytes). **B:** Bar graph represents the number of TUNEL+ chondrocytes per total chondrocytes ($n \geq 5$ /gp). * $p < 0.01$ vs. +/+ VEH; ** $p < 0.001$ vs. +/+ PTH. **C:** Bar graph represents the total number of chondrocytes per cartilage area ($n \geq 5$ /gp). Data is expressed as mean \pm SEM.

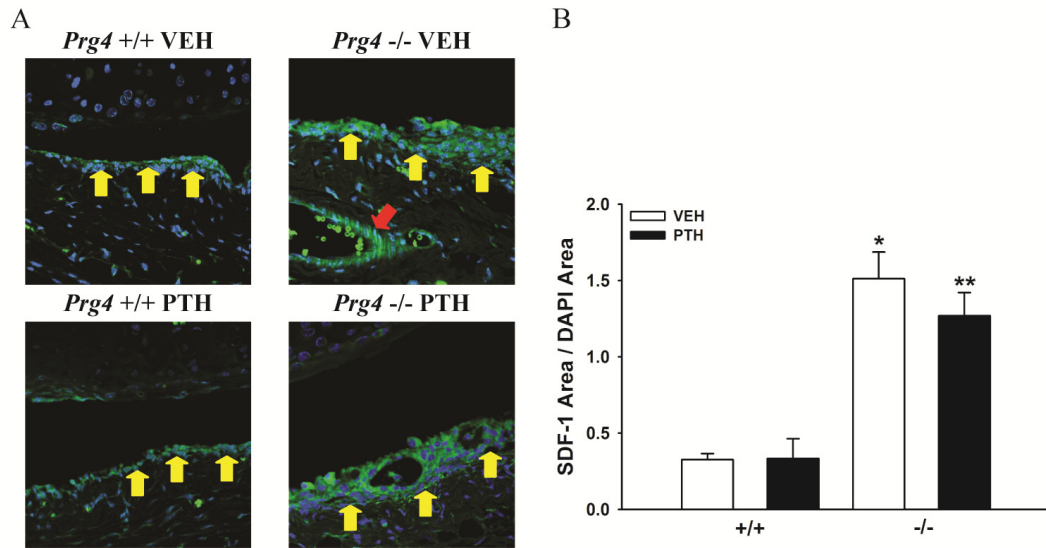


Figure 4.5. SDF-1 expression in the synovium. **A** and **B**: Sixteen week old *Prg4* mutant (-/-) and wildtype (+/+) mice were administered intermittent PTH (1-34) (50 μ g/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks, from 16-22 weeks. Stromal cell-derived factor-1 (SDF-1) immunofluorescence (IF) labeled sagittal knee sections were analyzed for SDF-1 expression in the synovium. **A**: Representative images of SDF-1 labeled synovium (60X) (SDF-1/Green and DAPI/Blue) (**yellow arrows** indicate SDF-1+ synovium and **red arrow** indicates SDF-1+ perivascular endothelial cells). **B**: Bar graph represents the SDF-1 area / DAPI area in the synovium (n=4-6/gp). * $p < 0.001$ vs. +/+ VEH; ** $p < 0.01$ vs. +/+ PTH. Data is expressed as mean \pm SEM.

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CHAPTER 5

CONCLUSIONS

The overall goal of this dissertation research was to elucidate the role of proteoglycan 4 (*Prg4*) in the actions of parathyroid hormone (PTH) in hematopoiesis, skeletal anabolism, and degenerating joints. The advancement of knowledge surrounding the mechanisms mediating PTH biologic actions is crucial since intermittent PTH therapy is approved for the treatment of osteoporosis and is actively under clinical investigations for local bone regenerative applications (1;2) as well as a potential stem cell therapy (3). Through *in vitro* and *in vivo* model systems relying on the *Prg4* null mutant mouse model (4), this body of work indicates that proteoglycan 4 supports the actions of PTH expanding HPCs, increasing bone mass, and protecting articular cartilage. In addition, this research extensively characterizing the *Prg4* mutant mouse has further defined the physiologic role of proteoglycan 4 in hematopoiesis, skeletogenesis, and joint protection.

The novel finding that PTH regulated gene expression similarly in bone and liver strongly suggests that the liver plays an important role in mediating the biologic actions of PTH. This is a notable discovery which may have broad implications for the advancement of PTH biology. The major contributions of this work (Chapter 2, 3, and 4) are summarized as follows.

Proteoglycan 4, a novel immunomodulatory factor, regulates parathyroid hormone actions on hematopoietic cells

This research utilizing the null *Prg4* mutant mouse provided for the first known *in vivo* study investigating the role of proteoglycan 4 as a physiologic regulator of hematopoiesis and PTH actions in hematopoiesis. While prior exogenous proteoglycan 4 administration studies have implicated proteoglycan 4 in the support of HPC expansion and megakaryopoiesis (5;6), the current *in vivo* investigation revealed that proteoglycan 4 is not a critical regulator of marrow HPC frequency or megakaryopoiesis. Interestingly, these studies demonstrated that proteoglycan 4 regulates marrow stromal cell-derived factor-1 (SDF-1) levels, immune cell populations, and PTH actions increasing marrow SDF-1 and HPCs.

The discovery of increased peripheral blood neutrophils and reduced marrow B-lymphocytic cell populations in adult *Prg4* mutant mice suggests that proteoglycan 4 is an immunomodulatory factor under physiologic conditions. While camptodactyly-arthropathy-coxa vara-pericarditis (CACP) patients have not been reported to spontaneously develop hematological abnormalities, we are unaware of any studies characterizing the bone marrow cell populations. Furthermore, while our study assessed hematopoiesis in an adult mouse model, investigations of CACP patients have been limited to the pediatric population (communications with Dr. Matthew Warman). The present study reveals that loss-of-function mutations in *PRG4* may result in altered immune cell populations which could have implications for immune response.

Similar to recent work demonstrating that interleukin-6 supports PTH actions on HPC expansion (7), the finding that PTH increased Lin⁻Sca-1⁺c-Kit⁺ cells more significantly in *Prg4* mutant vs. wildtype mice indicates that proteoglycan 4 supports the actions of PTH increasing HPCs. A better understanding of the mechanisms by which PTH increases marrow HPCs is critical for the safe and efficacious application of PTH as a stem cell therapy following bone marrow transplants.

While we speculated that alterations in marrow SDF-1 may be responsible for the differences in immune cells and PTH actions on HPCs in *Prg4* mutant mice, proteoglycan 4 actions on SDF-1 are unknown. Future studies are needed to elucidate the mechanism(s) by which proteoglycan 4 increases the concentration of SDF-1 in the marrow. Currently unavailable, mouse antibodies for the *Prg4* protein products (lubricin, SZP, MSF, and HAPO) would allow for immunolocalization studies to evaluate whether proteoglycan 4 interacts with SDF-1 to increase SDF-1 concentrations in the marrow. Based on recent work reporting that PTH increases SDF-1 in the ischemic heart via PTH actions inhibiting the enzyme dipeptidyl peptidase-IV (8), it is plausible that proteoglycan 4 could play a role stabilizing SDF-1 in the marrow.

The meniscal site of hematopoiesis in PTH-treated *Prg4* mutant mice was an unexpected finding. Taking into account that *Prg4* mutant mice have ectopic ossification in the joints (4), and PTH induced the formation of osteoclastic and osteoblastic cells lining the hematopoietic sites, it appears that the meniscal site of hematopoiesis in PTH-treated *Prg4* mutant mice is medullary hematopoiesis associated with advanced meniscal ossification. Future studies clarifying the pathophysiology of meniscal mineralization

and associated medullary hematopoiesis could have implications for preventing precocious joint failure in CACP patients.

Proteoglycan 4: a dynamic regulator of skeletogenesis and PTH skeletal anabolism

This original investigation of proteoglycan 4 actions in the skeleton revealed that proteoglycan 4 is a dynamic factor supporting skeletogenesis and PTH skeletal anabolism. The young *Prg4* mutant mouse model demonstrated that proteoglycan 4 supports endochondral bone formation and the attainment of peak trabecular bone mass in the developing skeleton. The reduced numbers of trabeculae, no difference in trabecular thickness, and lack of difference in bone cell numbers or activity in adult *Prg4* mutant mice suggest that proteoglycan 4 functions to support the formation of distinct trabeculae, but does not effect the remodeling of trabeculae.

The findings that young *Prg4* mutant mice have shortened interrupted columns of proliferative zone chondrocytes and decreased hypertrophic zone growth plate height suggest that proteoglycan 4 may contribute to the PTH-related protein (PTHrP) – indian hedgehog signaling feedback loop which supports chondrocyte proliferation while inhibiting chondrocyte maturation (9-11). Alternatively, proteoglycan 4 may impact endochondral bone formation by functioning as a structural component of the growth plate cartilage matrix. Future investigations are needed to further define the role of proteoglycan 4 in endochondral bone formation.

The decreased marrow and serum fibroblast growth factor 2 (FGF-2) levels in *Prg4* mutant mice are strong candidate regulators of proteoglycan 4 actions supporting trabecular bone formation. Findings in the present study which imply that liver derived FGF-2 contributes to circulating FGF-2 levels stress the need for investigations of the role that the liver plays in secreting FGF-2 into the circulation. Moreover the PTH induced increase in serum FGF-2 levels in osteopenic *Prg4* mutant mice and osteoporotic patients (12), highlights the need for future studies of the impact of circulating FGF-2 in the anabolic effects of PTH.

Since there were no differences in the cortical bone of young *Prg4* mutant mice, the cortical bone osteopenia in adult *Prg4* mutant mice appears to be due to altered skeletal loading secondary to disrupted joint function. While there have been controversial findings from investigations of PTH anabolic actions in rodent hindlimb immobilization models (13-16), the findings from the present study implying that PTH anabolic actions are blunted in *Prg4* mutant mice due to altered skeletal loading suggest that loading is a critical regulator of PTH actions increasing trabecular and cortical bone. This original study of the effects of PTH in an animal model having precocious joint failure validates the need for investigations of the efficacy of PTH anabolic therapy in patients afflicted by arthropathic joint conditions that alter skeletal loading.

The novel finding that PTH regulates PTH/PTHrP receptor (PPR), FGF-2, and *prg4* mRNA expression similarly in bone and liver suggests that in addition to well characterized actions in osteoblasts, PTH likely impacts skeletogenesis via actions in the liver. The generation of a hepatocyte specific PPR null mutant mouse would provide for

an effective model system to begin to elucidate the role that PPR signaling in the liver plays in PTH skeletal anabolism.

The impact of proteoglycan 4 and parathyroid hormone on articular cartilage degeneration

The investigation of the effect of proteoglycan 4 and the impact of PTH on articular cartilage in degenerating joints of adult *Prg4* mutant mice uncovered novel candidate mechanisms by which proteoglycan 4 protects articular cartilage. The irregularly contoured subchondral bone of the patellar groove in *Prg4* mutant mice reveals that *Prg4* loss-of-function mutation induced changes in articular joints are not limited to the synovium and cartilage. While it is unclear whether the irregularly contoured subchondral bone in *Prg4* mutant mice is due to proteoglycan 4 actions supporting skeletogenesis or protecting articular cartilage, the altered subchondral bone morphology likely contributes to the disruption of the articular cartilage in *Prg4* mutant joints.

The increased SDF-1 expression in the synovium of *Prg4* mutant mice, which appears to be secondary to synovial hyperplasia, is a notable discovery since there are currently no known non-mechanical mechanisms by which proteoglycan 4 protects articular cartilage. Based on recent *in vitro* work implicating supra-physiologic joint SDF-1 levels in articular chondrocyte cell death (17) and articular cartilage degradation

(18;19) we speculate that loss of superficial zone chondrocytes and increased cartilage degradation in *Prg4* mutant mice are attributed to increased joint SDF-1 levels.

The decreased chondrocyte apoptosis in the degenerating joints of *Prg4* mutant mice versus the healthy joints of *Prg4* wildtype mice strongly suggest that *Prg4* loss-of-function mutation results in chondrocyte cell autonomous alterations supporting cell survival. While it has been reported that *Prg4* mutant synoviocytes have cell intrinsic differences (4), this is the first known report of cell autonomous differences in the articular chondrocytes of *Prg4* mutant mice. Further studies elucidating the mechanisms by which *Prg4* regulates chondrocyte apoptosis will advance the understanding of proteoglycan 4 actions protecting articular cartilage.

Prior reports that systemic PTH administration has protective effects in degenerating joints are limited to a single meeting abstract (20). While further studies are needed, the minimal impact of subcutaneous PTH injection on the arthropathic joints of *Prg4* mutant mice suggests that systemic PTH does not have protective effects in degenerating joints deficient in proteoglycan 4.

Summary

This body of work supports prior studies that have attributed the anabolic effects of PTH to mechanisms that extend beyond direct PPR signaling in osteoblastic cells. While adult *Prg4* mutant mice had a blunted PTH-induced increase in trabecular and

cortical bone mass, *in vitro* and *in vivo* studies demonstrated that proteoglycan 4 is not a critical regulator of the anabolic actions of PTH in osteoblastogenesis or cellular level bone remodeling.

The broad scope of this dissertation work, assessing the impact of proteoglycan 4 in diverse PTH actions, suggests that proteoglycan 4 indirectly supports PTH anabolic effects via proteoglycan 4 actions in hematopoiesis and/or the joints (**Figure 5.1**). Based on studies implying that HPCs play a key role in regulating the anabolic effects of PTH in bone (21;22), the finding that proteoglycan 4 supports PTH actions expanding HPCs suggests that proteoglycan 4 actions in hematopoiesis may be linked to proteoglycan 4 actions supporting the anabolic effects of PTH. The decreased joint range of motion and animal mobility in *Prg4* mutant mice implies that proteoglycan 4 actions protecting joint function facilitate physiologic skeletal loading, which has been shown to be a critical regulator of PTH actions increasing trabecular and cortical bone (15;16). Future investigations rescuing the hematopoietic phenotype and/or altered skeletal loading in *Prg4* mutant mice will clarify the indirect mechanisms by which proteoglycan 4 supports PTH anabolic actions in the skeleton.

In addition to implicating hematopoietic cells and normal joint function as candidate regulators of PTH anabolic actions, findings in this thesis study demonstrating that PTH regulates gene expression similarly in bone and liver highlights the liver as a potential mediator of PTH biologic actions (**Figure 5.1**). In summary, this body of work strongly suggests that PTH biologic actions are mediated by diverse mechanisms which extend beyond direct PPR signaling in osteoblastic cells.

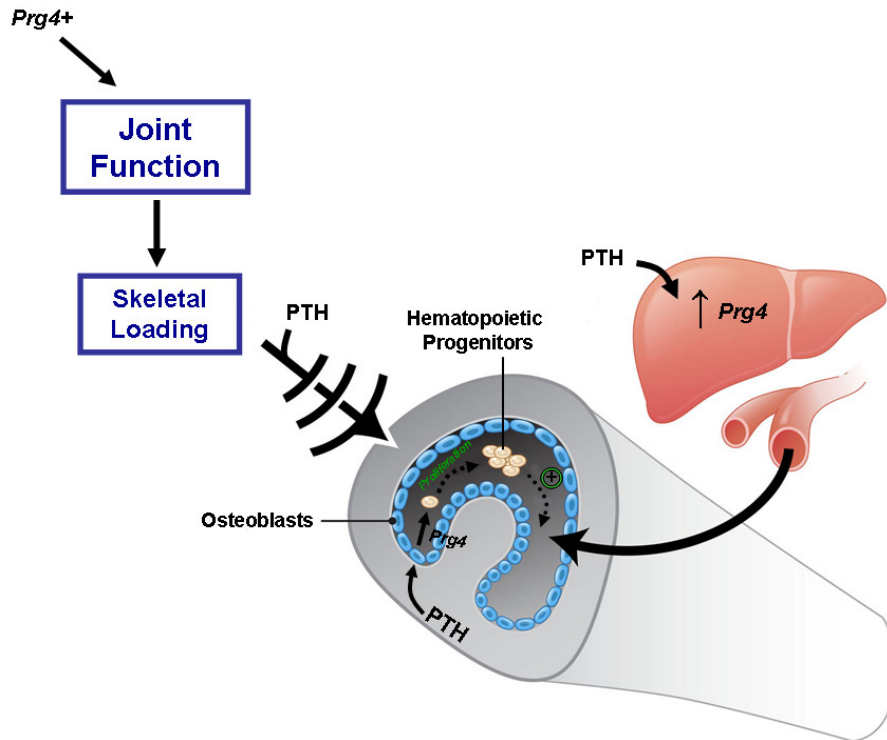


Figure 5.1. Proteoglycan 4 indirectly supports PTH anabolic actions. Proteoglycan 4 protects joint function, supports PTH actions increasing marrow hematopoietic progenitor cells, and is a PTH-responsive gene in the liver. These proteoglycan 4 effects are strong candidate regulators of the proteoglycan 4 actions supporting PTH anabolic effects increasing bone mass.

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