Metabolic, Endocrine, and Genitourinary Pathobiology

Proteoglycan 4, a Novel Immunomodulatory Factor, Regulates Parathyroid Hormone Actions on Hematopoietic Cells

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Proteoglycan 4 (PRG4), a critical protective factor in artic-ular joints, is implicated in hematopoietic progenitor cell expansion and megakaryopoiesis. *PRG4* loss-of-function mutations result in camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, which is characterized pri-marily by precocious joint failure. *PRG4* was identified as a novel parathyroid hormone (PTH) responsiveness gene in osteoblastic cells in bone, and was investigated as a potential mediator of PTH actions on hematopoiesis. Six-teen-week-old *Prg4*⁻/⁻ mutant and *Prg4*⁺/⁺ wild-type mice were treated daily with intermittent PTH (residues 1–34) or vehicle for 6 weeks. At 22 weeks of age, *Prg4* mutant mice had increased peripheral blood neutrophils and decreased marrow B220⁺ (B-lymphocytic) cells, which were normalized by PTH. The PTH-induced in-crease in marrow Lin⁻Sca-1⁺c-Kit⁺ (hematopoietic pro-genitor) cells was blunted in mutant mice. Basal and PTH-stimulated stromal cell-derived factor-1 (SDF-1) was decreased in mutant mice, suggesting SDF-1 as a candidate regulator of proteoglycan 4 actions on hematopoiesis *in vivo*. PTH stimulation of IL-6 mRNA was greater in mutant than in wild-type calvaria and bone marrow, suggesting a compensatory mechanism in the PTH-induced increase in marrow hematopoietic progenitor cells. In summary, pro-teoglycan 4 is a novel PTH-responsive factor regulat-ing immune cells and PTH actions on marrow hematopoi-etic progenitor cells. *(Am J Pathol 2011, 179:2431–2442; DOI: 10.1016/j.ajpath.2011.07.032)*

Parathyroid hormone (PTH), best known for its roles in cal-cium homeostasis and bone remodeling,[1] supports hema-topoietic progenitor cell (HPC) expansion and mobiliza-tion.[2–4] Although clinical investigations are assessing intermittent PTH administration as a potential stem cell ther-apy after bone marrow transplants,[5] the mechanisms me-diating PTH actions on hematopoiesis are poorly under-stood.

Stromal osteoblastic cells are the dominant cell type ex-pressing PTH/PTH-related protein (PTHrP) receptor (PPR) in the bone marrow. With the exception of specific PPR-expressing T-cell subpopulations,[6,7] PTH actions on hema-topoiesis are thought to be mediated via paracrine or jux-tacrine signaling from PPR-activated stromal osteoblastic cells.[1,2,8]

PPR signaling in stromal osteoblastic cells alters the expression of osteoclastic cytokines, most notably recep-tor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), which regulate the differentiation of hematopoietic cells to mature bone resorbing oste-oclastic cells.[8–12] More recent studies have focused on the role of PTH actions on HPCs. Calvi et al[2] demon-strated in mutant mice with osteoblast-specific constitu-tively active PPR that osteoblastic cells increase HPC expansion via Jagged1 ligand. Further investigation re-vealed that intermittent PTH administration in normal mice supported hematopoiesis through actions on early HPCs.[2] *Ex vivo* and *in vivo* PTH studies by Pirih et al[13] using the global IL-6 knockout mouse model demon-strated that IL-6 supports PTH expansion of HPCs.

The proteoglycan 4 gene (human *PRG4*; murine *Prg4*), which has been implicated in HPC expansion[14–16] and megakaryopoiesis,[17–19] was identified as a novel PTH-

Table 1. *PRG4* Protein Products

Protein product	Isolation	Expression	Known functions
Lubricin[23]	Bovine synovial fluids	Synoviocytes	Boundary lubrication of synovial joints
Superficial zone protein (SZP)[24]	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

responsive gene in bone.[20,21] The *PRG4* gene encodes an approximately 345-kDa proteoglycan, consisting of 1404 amino acids spanning 12 exons. *PRG4* is expressed across skeletal and nonskeletal tissues, with highest relative levels of expression in articular joints, liver, and bone.[22,23] The four isolated *PRG4* protein products [ie, lubricin, superficial zone protein (SZP), hemangiopoietin (HAPO), and megakaryocyte stimulating factor (MSF)] are secreted glycoproteins that have been implicated in articular joint protection,[23,24] HPC expansion,[14] and megakaryopoiesis[17] (Table 1). A proteoglycan 4 receptor has not yet been identified.[14,23] Loss-of-function mutations in *PRG4* result in the human autosomal recessive disorder known as camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP), which is characterized by early-onset joint failure and osteopenia. CACP patients have not been reported to spontaneously develop hematological disorders.[25]

Although selected studies have reported that HAPO supports HPC expansion[14–16] and that MSF regulates megakaryopoiesis,[17–19] the actions of proteoglycan 4 on hematopoiesis are unclear. The purpose of the present study was to investigate the unexplored role of proteoglycan 4 as a regulator of physiological hematopoiesis and PTH actions on hematopoiesis in the mature skeleton. Using the *Prg4*-null mouse model,[23] we report the first known study of *Prg4* actions on hematopoiesis *in vivo*.

Materials and Methods

C57BL/6 Wild-Type Mice, Single PTH Administration

Sixteen-week-old C57BL/6 wild-type mice were administered a single subcutaneous injection of recombinant human PTH residues 1 to 34 [PTH(1–34); 1 μg/g] (Bachem, Torrance, CA) or vehicle (0.9% NaCl) and then were sacrificed 4, 8, or 12 hours later; bone marrow was harvested for gene expression analysis. All animal studies were approved by the University Committee on the Use and Care of Animals (UCUCA) of the University of Michigan, and animals were maintained in accordance with approved UCUCA research protocols.

Primary Calvarial Osteoblastic Cell Cultures, Single PTH Administration

Primary osteoblasts were isolated from calvariae harvested from 7- to 14-day-old C57BL/6 wild-type mice.

Briefly, calvariae were dissected, isolated from periosteum, and subjected to sequential digestion for 10, 20, and 60 minutes in α-modified minimum essential medium (Invitrogen, Carlsbad, CA) 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 $5 \times 10^4/cm^2$ in α-modified minimum essential medium with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine. Cells were induced to differentiate by supplementing medium with 50 μg/mL ascorbic acid.

Cultures at day 5 (d5) were administered a single recombinant human PTH(1–34) (10 nmol/L) (Bachem) or vehicle (4 mmol/L HCl/0.1%BSA) treatment and were harvested 8, 12, or 24 hours later for gene expression analysis. In another experimental protocol, d1, d5, d11, and d14 cultures were administered a single recombinant human PTH(1–34) (10 nmol/L) (Bachem) or vehicle (4 mmol/L HCl/0.1%BSA) treatment and were harvested 4 hours later for gene expression analysis.

Background of Prg4$^{-/-}$ Mutant Mice

Prg4$^{-/-}$ mutant mice with a mutant *Prg4* allele generated by homologous recombination in 129Sv/Ev-derived embryonic stem cells were generously provided by Matthew Warman (Harvard University).[23] Mutant mice were backcrossed from the 129Sv/Ev genetic background to the C57BL/6 genetic background. A PCR-based assay was used to genotype the mice, as described previously.[23]

Prg4$^{-/-}$ Mutant Mice, Single PTH Administration

At 16 weeks of age, *Prg4*$^{-/-}$ mutant and *Prg4*$^{+/+}$ wild-type littermate mice were administered a single subcutaneous injection of recombinant human PTH(1–34) (1 μg/g) (Bachem) or vehicle (0.9% NaCl) and then were sacrificed 1, 4, 8, or 12 hours later; bone marrow and calvariae were harvested for gene expression analysis.

Prg4$^{-/-}$ Mutant Mice, 6 Weeks Daily Intermittent PTH Administration

In an intermittent PTH experimental protocol, 16-week-old *Prg4*$^{-/-}$ mutant and *Prg4*$^{+/+}$ wild-type 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, to 22 weeks of age. Mice were sacrificed 24 hours after the final injection, and tissues were harvested for analyses.

Quantitative Real-Time PCR

Bone marrow was directly flushed from femur and tibia with TRIzol reagent (Invitrogen) using a 3-mL syringe and 22G ½-inch (~12.7-mm length) needle. Calvariae were flash-frozen, pulverized, and homogenized in TRIzol reagent. Calvarial osteoblast cultures were washed three times with 1× PBS, and TRIzol was directly applied to cultures. In each case, RNA was isolated according to manufacturer's directions, and total RNA was quantified. Double-stranded cDNA was synthesized from 1.0 μg of RNA, using random hexamers and MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). cDNA was amplified using the TaqMan universal PCR master mix with TaqMan gene expression-specific primers and 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 were determined using the standard curve method or the comparative C_t method.[26]

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 a Hemavet 1500FS system (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 (FCSB) (1× PBS, 2% fetal bovine serum, 2 mmol/L 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 from the sectioned mid-diaphysis with FCSB. Spleens were incised lengthwise, placed on a cell strainer, and disassociated with a 1-mL syringe plunger while FCSB was being delivered at the same time. Cell suspensions were homogenized, strained, centrifuged, and resuspended; cell counts were then performed. Three million cells were suspended in 0.5 mL FCSB, incubated with fluorochrome-conjugated antibodies (BD Biosciences) for 30 minutes at 4°C, and then were washed, centrifuged, and resuspended in 1 mL FCSB containing 7-AAD (Invitrogen) for analysis. All antibodies were 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$^+$ cells was assessed in spleens. Samples were analyzed using a FACSCalibur flow cytometer and CellQuest Pro software version 5.1 (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 FCSB, incubated with FITC-conjugated CD41 antibody for 30 minutes at 4°C, rinsed with 1× PBS, centrifuged, and fixed in 1 mL 1% paraformaldehyde overnight at 4°C. Cells were centrifuged, washed with 1× PBS, resuspended in 1 mL propidium iodide staining solution (1× PBS, 4 mmol/L sodium citrate, 0.1% Triton X-100, 50 μg/mL propidium iodide, and 100 μg/mL RNase A), and incubated for 1 hour at 37°C. A minimum of 10,000 gated cells were analyzed for each sample. Samples were analyzed using a FACSCalibur flow cytometer and CellQuest Pro software version 5.1 (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 in modified methylmethacrylate. Frontal serial sections (4 μm) were cut with vertical bed microtomes (Jung 2065 and 2165; Leica Microsystems, Bannockburn, IL) and affixed to slides precoated 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 semiautomatically with a Nikon light microscope and the OsteoMeasure trabecular analysis system (OsteoMetrics, 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-thick serial sections were cut and stained. H&E staining was performed on all tissue sections.

SDF-1 immunofluorescence analysis was performed in proximal tibia sections. Samples were deparaffinized with mixed xylenes, rehydrated in graded ethanols, and then briefly washed with PBT (PBS-0.2% Triton X-100) and blocked for 30 minutes with Image-iT FX signal enhancer (Invitrogen). SDF-1 immunofluorescence analysis was performed using a Zenon AlexaFluor 488 rabbit labeling kit (Invitrogen) and SDF-1 α rabbit polyclonal antibody (ab25117; Abcam, Cambridge, MA). After overnight incubation at 4°C with 1 μg/100 μL dilution of fluorescence-labeled SDF-1 α rabbit polyclonal antibody (Abcam), sections were washed with PBT, mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), and coverslipped. Images were acquired with an Olympus FV-500 confocal microscope. Image analysis of SDF-1 immunofluorescence in proximal tibia trabecular bone (secondary spongiosa) was performed using Image Pro Plus version 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 a 22G ½-inch needle. The marrow suspension was homogenized and cell counts performed. Cell suspensions were centrifuged at $81.6 \times g$ for 5 minutes, supernatants were collected, stored at $-80°C$, and freeze-thawed once. SDF-1 levels in the bone marrow supernatant were analyzed by antibody sandwich enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol, with a detection range of 62.5 to 10,000 pg/mL. SDF-1 levels were normalized to marrow cell numbers.

Statistical Analysis

Analysis of variance and unpaired *t*-test were performed using GraphPad Instat software version 3.10 (GraphPad Software, San Diego, CA). Statistical significance was set at $P \leq 0.05$. Data are expressed as means $\pm$ SEM.

Results

Single PTH Administration Increases Prg4 mRNA Levels in Bone

Quantitative real-time PCR studies demonstrated that *Prg4* is a novel PTH-responsiveness gene in bone, both *in vivo* and *in vitro* (Figure 1, A–C). A single subcutaneous injection of PTH(1–34) (1 μg/g) in 16-week-old C57BL/6 wild-type mice significantly increased *Prg4* mRNA in bone marrow at 4 and 8 hours after injection (Figure 1A). Moreover, a single PTH(1–34) administration (10 nmol/L) to primary calvarial osteoblastic cell cultures significantly up-regulated *Prg4* mRNA at 8, 12, and 24 hours after treatment (Figure 1B).

Prg4 mRNA expression was evaluated in d1, d5, d11, and d14 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 (Figure 1C). At 4 hours, a single PTH administration significantly increased *Prg4* mRNA at all stages of differentiation. Relative to vehicle-control samples, PTH significantly increased *Prg4* mRNA at day 1 (1.5-fold), day 5 (2.8-fold), day 11 (5.3-fold), and day 14 (24.4-fold) (Figure 1C). Osteocalcin gene expression studies validated the maturation of osteoblastic cells in culture over time (Figure 1D), as we have previously reported.[27]

Bone Marrow, Liver, and Spleen Morphology

Tibia, liver, and spleen were isolated after 6 weeks of PTH or vehicle treatment (ie, 22-week-old mice) for histological evaluation of hematopoietic organs (Figure 2, A–C). Proximal tibia marrow morphology was similar in 22-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice (Figure 2A). Likewise, there were no morphological differences found in 22-week-old $Prg4^{-/-}$ liver (Figure 2B) or spleen (Figure 2C) relative to $Prg4^{+/+}$ littermates, and no evidence of extramedullary hematopoiesis in these organs.

Bone Marrow IL-6, Thrombopoietin, and SDF-1 mRNA in Prg4$^{-/-}$ Mice

Based on previous reports implicating the *Prg4* protein products HAPO and MSF in HPC expansion[14–16] and megakaryopoiesis,[17–19] we evaluated the expression of several genes that are dual regulators of HPCs and megakaryopoiesis. Sixteen-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice were administered a single subcutaneous
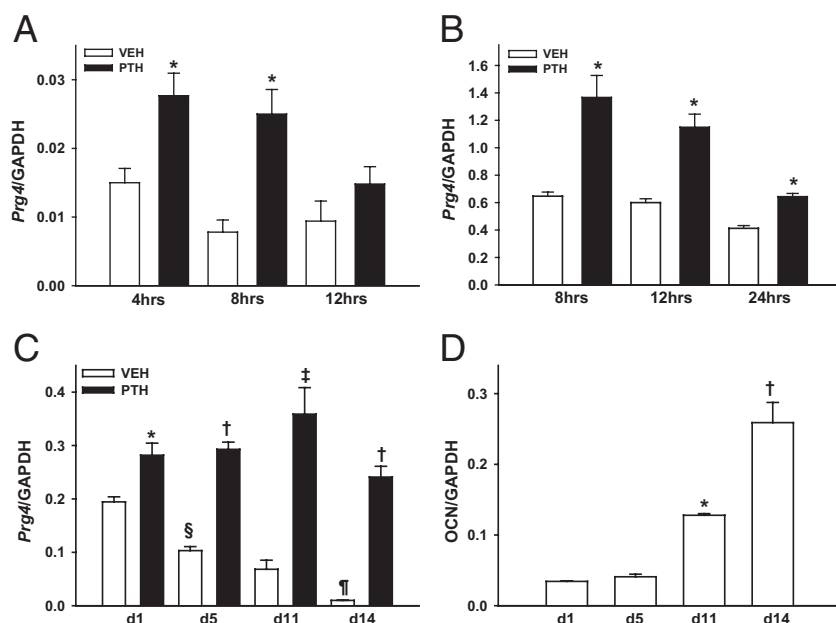

Figure 1. PTH regulation of *Prg4* mRNA. **A:** Sixteen-week-old C57BL/6 wild-type mice were administered a single subcutaneous injection of PTH(1–34) (1 μg/g) or vehicle (VEH) (0.9% NaCl) control and then were sacrificed 4, 8, or 12 hours later; bone marrow was harvested for *Prg4* mRNA expression analysis (*n* = 5 per group). *$P < 0.05$ versus VEH. **B:** C57BL/6 wild-type mouse-derived calvarial osteoblast cultures were administered a single treatment of PTH (10 nmol/L) or vehicle (VEH) (4 mmol/L HCl/0.1% SA) and were harvested 8, 12, or 24 hours later for *Prg4* mRNA expression analysis (*n* = 7 per group). *$P < 0.01$ versus VEH. **C:** C57BL/6 wild-type mouse-derived calvarial osteoblast cultures, 1, 5, 11, and 14 days after plating, were administered a single treatment of PTH (10 nmol/L) or vehicle (VEH) (4 mmol/L HCl/0.1%BSA) treatment and were harvested 4 hours later for *Prg4* mRNA expression analysis (*n* = 3 per group). *$P < 0.05$, PTH versus VEH; †$P < 0.001$, PTH versus VEH; ‡$P < 0.01$, PTH versus VEH; §$P < 0.01$, d5-VEH versus d1-VEH; ¶$P < 0.05$, d14-VEH versus d11-VEH. **D:** From the same cultures as in **C**, osteocalcin (OCN) mRNA expression was assessed as a marker of osteoblast differentiation. *$P < 0.001$, d11 versus d5; †$P < 0.05$, d14 versus d11. RNA was isolated and quantitative real-time PCR was performed to assess *Prg4* mRNA and OCN mRNA expression (standardized to GAPDH levels). Relative quantification of data generated was determined using the standard curve method. Data are expressed as means $\pm$ SEM.
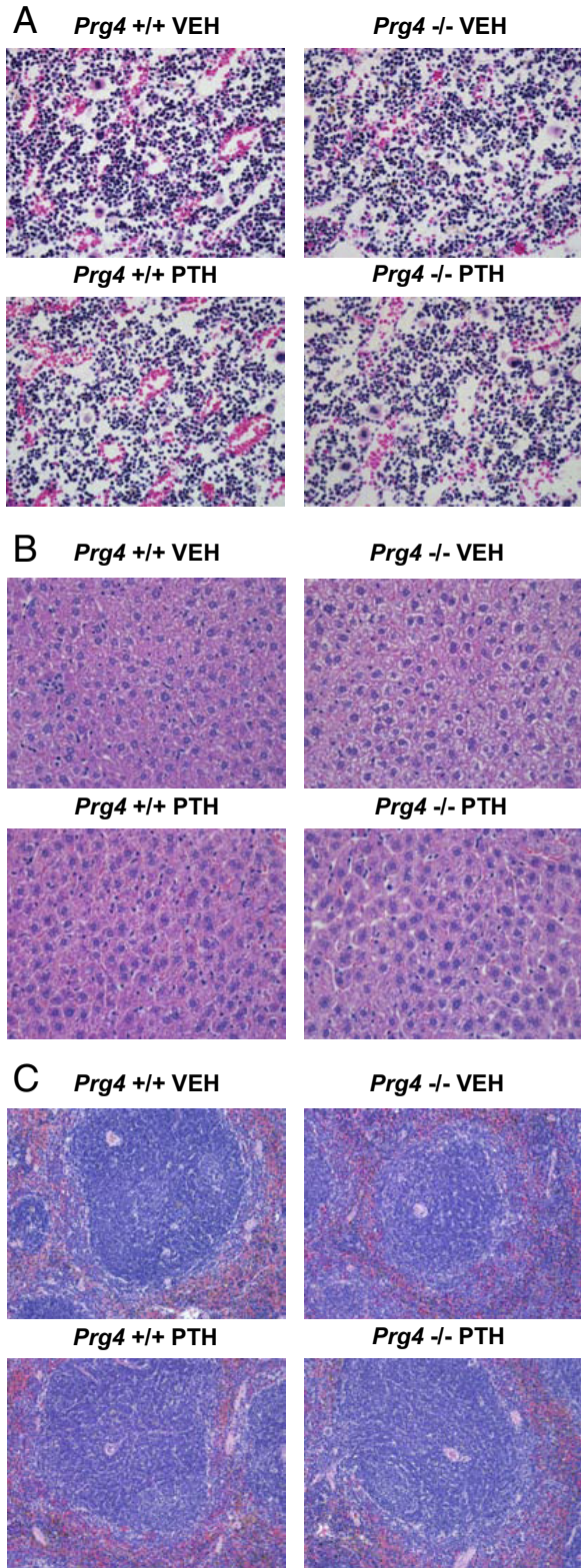
Figure 2. Histological assessment of bone marrow, liver, and spleen. $Prg4^{-/-}$ mutant and $Prg4^{+/+}$ wild-type mice were administered intermittent PTH(1–34) (50 μg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16 weeks to 22 weeks. Tibia, liver, and spleen were isolated from 22-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice for histological evaluation. Images are representative images of H&E-stained proximal tibia bone marrow sections ($n \geq 10$ per group) (**A**), liver sections ($n \geq 5$ per group) (**B**), and spleen sections ($n \geq 5$ per group) (**C**). Original magnification: ×40 (**A** and **B**); ×20 (**C**).

injection of PTH(1–34) (1 μg/g) or vehicle (0.9% NaCl) control and then were sacrificed 1, 4, 8, or 12 hours later; bone marrow total RNA was isolated, and quantitative real-time PCR was performed to assess IL-6, TPO, and SDF-1 mRNA expression.

IL-6 is a pleiotropic factor with diverse biological roles, including the support of hematopoiesis, lymphopoiesis,[28,29] and megakaryopoiesis.[30,31] Marrow IL-6 mRNA levels were significantly lower in control 16-week-old $Prg4^{-/-}$ mice and were consistently expressed at 60% of the levels detected in $Prg4^{+/+}$ littermates (Figure 3A). *IL-6* is an immediate early-response gene up-regulated in bone *in vitro* and *in vivo* by PTH at 30 minutes to 1 hour after administration.[32–34] IL-6 mRNA levels were significantly increased at 1 hour after PTH injection in 16-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ marrow, returning to baseline at 4 hours. Relative to vehicle-control samples, PTH injection increased IL-6 mRNA to a significantly greater extent at 1 hour in $Prg4^{-/-}$ marrow (13-fold) than in $Prg4^{+/+}$ marrow (fivefold) ($P < 0.01$) (Figure 3A).

IL6 gene expression studies were performed in calvariae harvested from untreated and single PTH-treated 16-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice to characterize the cellular target mediating the exacerbated PTH-induced increase in marrow IL-6 mRNA in $Prg4^{-/-}$ mice. Relative to controls, PTH injection induced a significantly greater increase in calvarial IL-6 mRNA in $Prg4^{-/-}$ (132-fold) than in $Prg4^{+/+}$ mice (45-fold) at 1 hour after treatment ($P < 0.05$), substantiating an osteoblast target (Figure 3B).

TPO, the primary factor regulating megakaryopoiesis, has been shown to support HPCs.[35] Marrow TPO mRNA expression was similar in control 16-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice, and PTH did not alter marrow TPO mRNA levels in $Prg4^{-/-}$ or $Prg4^{+/+}$ mice (Figure 3C).

SDF-1, a chemokine expressed at high levels by bone marrow stromal osteoblastic cells, regulates the homing and mobilization of marrow HPCs,[36,37] megakaryocyte progenitor cells,[38] and immune cell populations.[39,40] Although intermittent PTH treatment has been reported to increase SDF-1 mRNA expression in bone, *in vitro* and *in vivo*,[41] little is known regarding the temporal effects of PTH on SDF-1 mRNA expression. At 4 hours after PTH injection, marrow SDF-1 mRNA levels were reduced similarly in 16-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice. At 12 hours after PTH injection, marrow SDF-1 mRNA was significantly increased in $Prg4^{+/+}$ mice but PTH notably failed to increase marrow SDF-1 mRNA in $Prg4^{-/-}$ mice (Figure 3D). Gene expression studies in calvariae harvested from untreated and single PTH-treated 16-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice demonstrated no change in SDF-1 mRNA expression at 12 hours after treatment (data not shown).

$Prg4^{-/-}$ Mice Have Altered Immune Cell Populations, Normalized by PTH

Whole blood was collected from 22-week-old PTH-treated and vehicle-treated $Prg4^{-/-}$ and $Prg4^{+/+}$ mice, and complete blood count studies were performed to
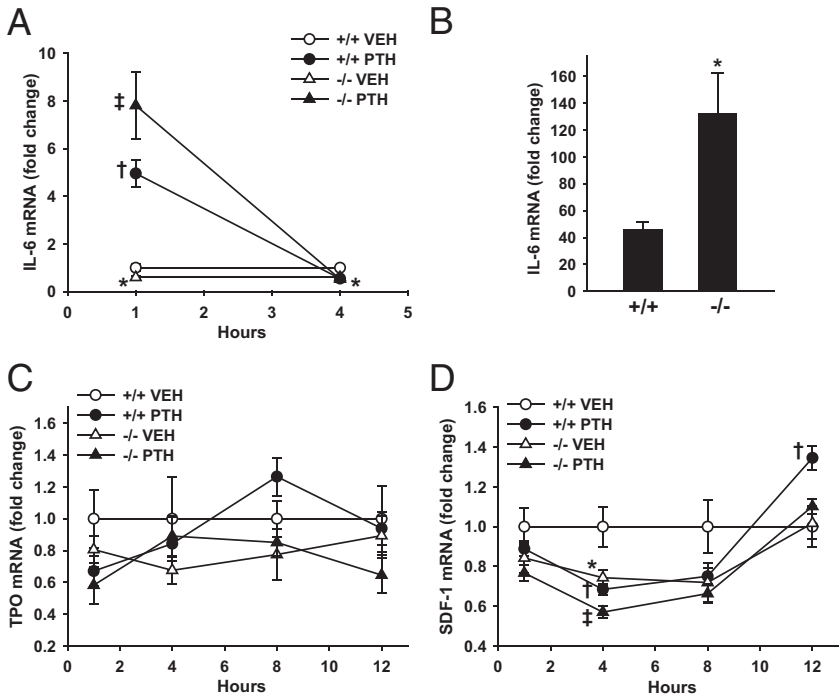
Figure 3. PTH regulation of gene expression. **A**, **C**, and **D**: Sixteen-week-old *Prg4*$^{-/-}$ mutant and *Prg4*$^{+/+}$ wild-type mice were administered a single subcutaneous injection of PTH(1–34) (1 μg/g) or vehicle (VEH) (0.9% NaCl) control and then were sacrificed 1, 4, 8, or 12 hours later; bone marrow was harvested for gene expression analysis ($n = 5$ per group). RNA was isolated and quantitative real-time PCR was performed to assess mRNA expression of IL-6 (**A**), TPO (**C**), and SDF-1 (**D**), standardized to GAPDH levels. Relative quantification of data was determined using the comparative C_t method. **B:** Sixteen-week-old *Prg4*$^{-/-}$ and *Prg4*$^{+/+}$ mice were untreated or administered a single subcutaneous injection of PTH(1–34) (1 μg/g) and then were sacrificed 1 hour later; calvariae were harvested for gene expression analysis ($n = 5$ per group). RNA was isolated and quantitative real-time PCR was performed to assess IL-6 mRNA expression (standardized to GAPDH levels). Values are expressed as treatment over no treatment control within treatment groups. **A:** Marrow IL-6 mRNA fold change at 1 and 4 hours after PTH injection. *$P < 0.05$, $-/-$ VEH versus $+/+$ VEH; $^{†}P < 0.001$, $+/+$ PTH versus $+/+$ VEH; $^{‡}P < 0.001$, $-/-$ PTH versus $-/-$ VEH. **B:** Calvarial IL-6 mRNA fold change at 1 hour after PTH injection. *$P < 0.05$, $-/-$ PTH versus $+/+$ PTH. **C:** Marrow TPO mRNA fold change at 1, 4, 8, and 12 hours after PTH injection. **D:** Marrow SDF-1 mRNA fold change at 14, 8, and 12 hours after PTH injection. *$P < 0.05$, $-/-$ VEH versus $+/+$ VEH; $^{†}P < 0.05$, $+/+$ PTH versus $+/+$ VEH; $^{‡}P < 0.01$, $-/-$ PTH versus $-/-$ VEH. Data are expressed as means ± SEM.

assess peripheral blood cell populations. There were no differences in red blood cell values or platelet numbers between *Prg4*$^{-/-}$ and *Prg4*$^{+/+}$ mice (see Supplemental Table S1 at *http://ajp.amjpathol.org*). *Prg4*$^{-/-}$ mice had altered peripheral white blood cells, including increased percentages of neutrophils and decreased percentages of lymphocytes (Figure 4, A and B; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). Unexpectedly, PTH treatment normalized the frequency of peripheral blood neutrophils and lymphocytes in *Prg4*$^{-/-}$ mice to

the levels of *Prg4*$^{+/+}$ mice (Figure 4, A and B; see also Supplemental Table S1 at *http://ajp.amjpathol.org*). Analysis of absolute cell numbers revealed that *Prg4*$^{-/-}$ mice had increased numbers of neutrophils that were normalized by PTH (Figure 4, C and D; see also Supplemental Table S1 at *http://ajp.amjpathol.org*).

To elucidate whether differences in peripheral blood immune cells reflected changes in the marrow, femoral bone marrow was harvested from 22-week-old *Prg4*$^{-/-}$ and *Prg4*$^{+/+}$ mice for flow cytometric analysis of
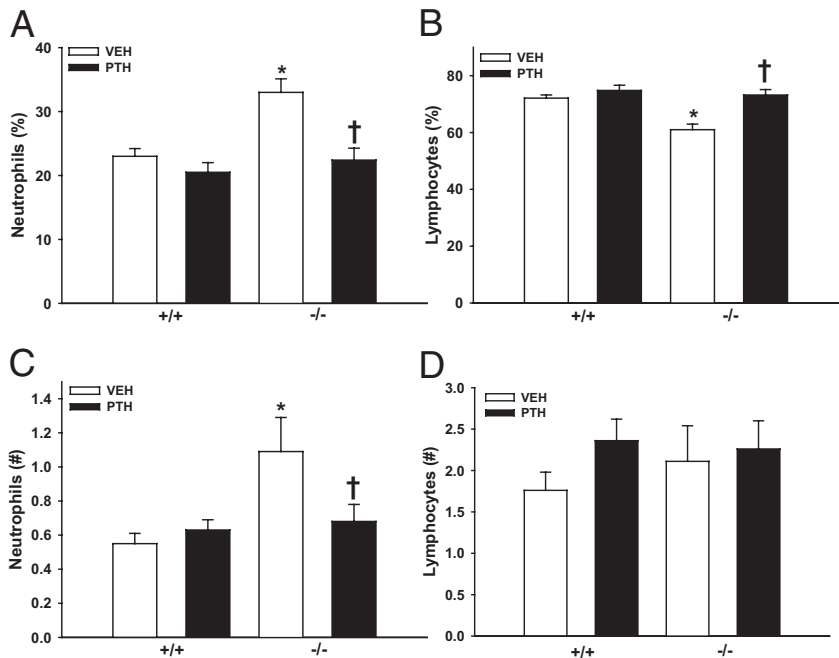

Figure 4. Peripheral blood cell analyses. **A–D:** *Prg4*$^{-/-}$ mutant and *Prg4*$^{+/+}$ wild-type mice were administered intermittent PTH(1–34) (50 μg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injection daily for 6 weeks, from age 16 weeks to 22 weeks. Peripheral blood was isolated via cardiac puncture from 22-week-old *Prg4*$^{-/-}$ and *Prg4*$^{+/+}$ mice and complete blood count analysis (automated differential) was performed to assess peripheral blood cell populations. $n \geq 10$ per group. **A:** Percentage of neutrophils. *$P < 0.001$ versus $+/+$ VEH; $^{†}P < 0.01$ versus $-/-$ VEH. **B:** Percentage of lymphocytes. *$P < 0.001$ versus $+/+$ VEH; $^{†}P < 0.001$ versus $-/-$ VEH. **C:** Number of neutrophils. *$P < 0.01$ versus $+/+$ VEH; $^{†}P < 0.05$ versus $-/-$ VEH. **D:** Number of lymphocytes. Data are expressed as means ± SEM.
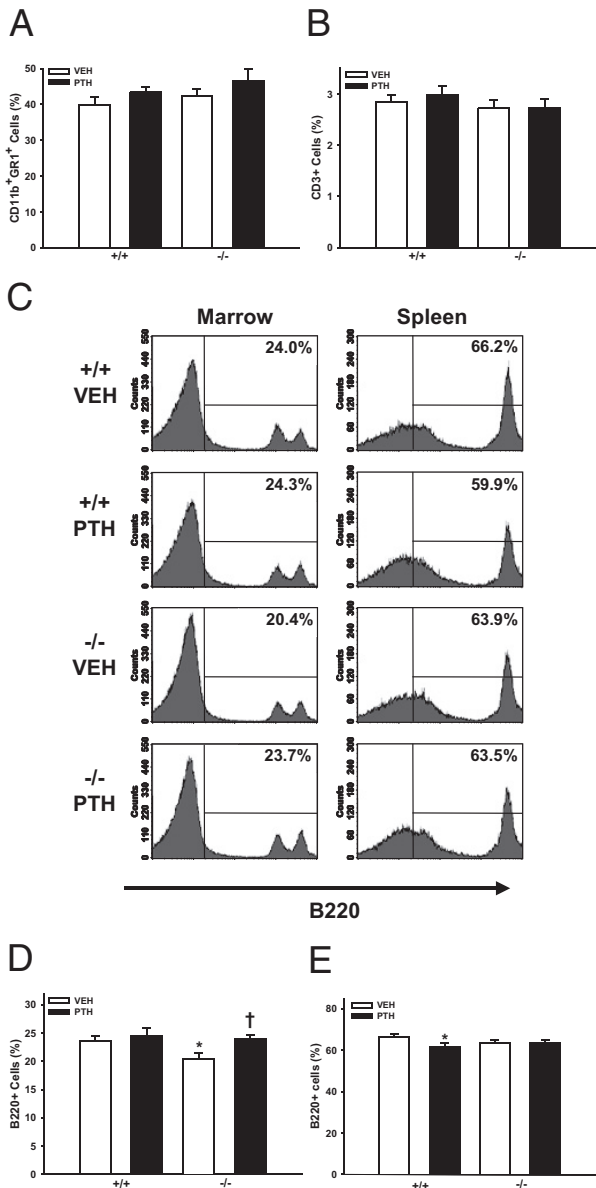
Figure 5. Bone marrow and spleen immune cell analyses. **A–D:** $Prg4^{-/-}$ mutant and $Prg4^{+/+}$ wild-type mice were administered intermittent PTH(1–34) (50 μg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16 weeks to 22 weeks. The 22-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice were sacrificed and femur bone marrow cells were isolated for flow cytometric analysis of CD11b⁺GR1⁺ (immature myeloid) cells ($n ≥ 9$ per group) (**A**), CD3⁺ (T-lymphocytic) cells ($n ≥ 9$ per group) (**B**), and B220⁺ (B-lymphocytic) cells ($n ≥ 9$ per group) (**C** and **D**). **C** and **E:** Spleen cells were isolated for flow cytometric analysis of B220⁺ cells ($n ≥ 10$ per group). **C:** B220⁺ marrow and spleen cells. $*P < 0.05$ versus $+/+$ VEH; $^{†}P < 0.05$ versus $-/-$ VEH. Data are expressed as means ± SEM. Percentages shown are relative to total cells in marrow (**A**, **B**, and **D**) or spleen (**E**).

CD11b⁺GR1⁺ (immature myeloid) cells, CD3⁺ (T-lymphocytic) cells, and B220⁺ (B-lymphocytic) cells. The frequency of marrow CD11b⁺GR1⁺ cells and CD3⁺ cells did not differ between $Prg4^{-/-}$ and $Prg4^{+/+}$ mice (Figure 5, A and B; see also Supplemental Figure S1 at *http://ajp.amjpathol.org*). Similar to the peripheral blood lymphocyte findings, $Prg4^{-/-}$ mice had a reduced percentage of marrow B220⁺ cells, which were normalized to levels of

$Prg4^{+/+}$ mice with PTH treatment (Figure 5, C and D). Based on differences in peripheral blood lymphocytes and marrow B-lymphocytic cells, the frequency of B220⁺ cells was assessed in the spleen, a center for B-lymphopoiesis. The frequency of B220⁺ cells in spleen was similar in 22-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice (Figure 5, C and 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.[42] 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 Have Decreased Expansion of Marrow HPCs Induced by PTH

Recombinant HAPO administration increased marrow HPCs in normal mice and accelerated marrow hematopoietic reconstitution in mice subjected to irradiation[14] or high-dose chemotherapy.[15] To investigate the effect of proteoglycan 4 on marrow HPCs, we assessed the frequency of marrow Lin⁻Sca-1⁺c-Kit⁺ (hematopoietic progenitor) cells in 22-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice by flow cytometric analysis (Figure 6). The percentage of marrow Lin⁻Sca-1⁺c-Kit⁺ cells was similar in vehicle-treated $Prg4^{-/-}$ and $Prg4^{+/+}$ mice. PTH increased the frequency of Lin⁻Sca-1⁺c-Kit⁺ cells in both $Prg4^{-/-}$ and $Prg4^{+/+}$ marrow; however, this increase was blunted in $Prg4^{-/-}$ mice relative to $Prg4^{+/+}$ littermates (Figure 6). These data suggest that proteoglycan 4 supports PTH actions increasing marrow HPCs.

Marrow Megakaryopoiesis Is Similar in $Prg4^{-/-}$ and $Prg4^{+/+}$ Mice

Although 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 effect of proteoglycan 4 on megakaryopoiesis, the frequency of marrow CD41⁺ (megakaryopoietic) cells and megakaryocyte ploidy (a relative measure of megakaryocyte maturity) were evaluated in 22-week-old $Prg4^{-/-}$ versus $Prg4^{+/+}$ mice via flow cytometric analysis. The frequency of marrow CD41⁺ (megakaryopoietic) cells and megakaryocyte ploidy were similar in $Prg4^{-/-}$ and $Prg4^{+/+}$ mice (see Supplemental Figure S2, A and B at *http://ajp.amjpathol.org*). Numbers of megakaryocytes per marrow area in proximal tibia sections were similar in 22-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice (see Supplemental Figure S2, C and D, at *http://ajp.amjpathol.org*). These findings indicate that proteoglycan 4 is not a critical regulator of marrow megakaryopoiesis *in vivo*.
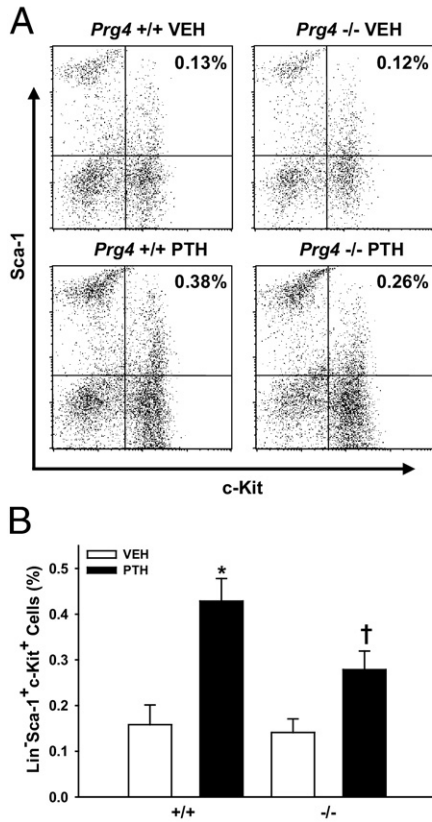
Figure 6. Bone marrow hematopoietic progenitor cell analyses. $Prg4^{-/-}$ mutant and $Prg4^{+/+}$ wild-type mice were administered intermittent PTH(1–34) (50 μg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16 weeks to 22 weeks. Femur bone marrow cells were isolated from 22-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice for flow cytometric analysis of $Lin^-Sca-1^+c-Kit^+$ (hematopoietic progenitor) cells ($n \geq 6$ per group). **A:** Representative dot plots of FITC/c-Kit (x axis) versus PE/Sca-1 (y axis) (gated for Lin^- cell population). **B:** Percentage of $Lin^-Sca-1^+c-Kit^+$ cells, relative to total marrow cells. *$P < 0.01$ versus +/+ VEH; †$P < 0.05$ versus −/− VEH & +/+ PTH. Data are expressed as means ± SEM.

Prg4$^{-/-}$ Mice Have Altered Marrow IL-6 mRNA and SDF-1 Protein

Marrow IL-6 mRNA expression was measured in 22-week-old PTH-treated and vehicle-treated $Prg4^{-/-}$ and $Prg4^{+/+}$ mice (Figure 7A), because IL-6 mRNA levels in control 16-week-old $Prg4^{-/-}$ mice were decreased, relative to $Prg4^{+/+}$ littermates. Similar to the 16-week-old $Prg4^{-/-}$ mice, control 22-week-old $Prg4^{-/-}$ mice had decreased marrow IL-6 mRNA levels, compared with $Prg4^{+/+}$ littermates (Figure 7A). These data suggest that $Prg4$ signaling supports basal IL-6 mRNA expression in the bone marrow.

Based on the findings that 22-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 22-week-old $Prg4^{-/-}$ versus $Prg4^{+/+}$ mice. Quantitative real-time PCR analysis demonstrated similar marrow SDF-1 mRNA levels in $Prg4^{-/-}$ and $Prg4^{+/+}$ mice (Figure 7B), which indicates that $Prg4$ does not affect
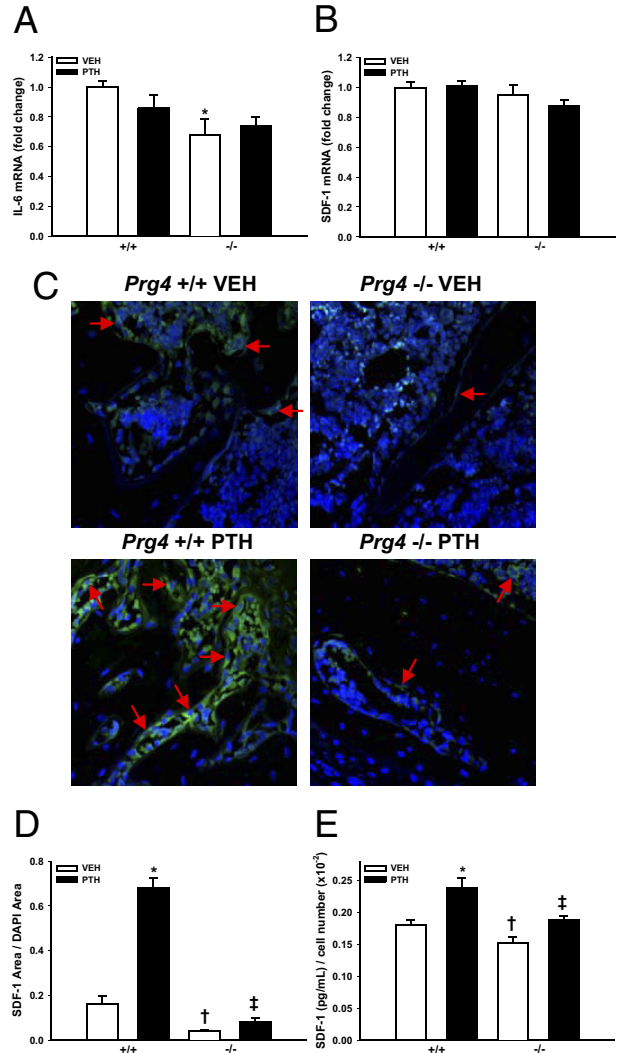

Figure 7. Bone marrow IL-6 and SDF-1 expression. **A–E:** $Prg4^{-/-}$ mutant and $Prg4^{+/+}$ wild-type mice were administered intermittent PTH(1–34) (50 μg/kg) or vehicle (VEH) (0.9% NaCl) control subcutaneous injections daily for 6 weeks, from age 16 weeks to 22 weeks. **A** and **B:** Bone marrow was harvested from 22-week-old $Prg4^{-/-}$ and $Prg4^{+/+}$ mice ($n = 5$ per group) for quantitative real-time PCR analysis of IL-6 (**A**) and SDF-1 (**B**) mRNA expression, standardized to GAPDH levels. Relative quantification of data was performed using the comparative C_t method. **A:** Marrow IL-6 mRNA expression. *$P < 0.05$ versus +/+ VEH. **B:** Marrow SDF-1 mRNA expression. **C** and **D:** SDF-1 immunofluorescence analysis was performed on proximal tibia (secondary spongiosa) sections of 22-week-old mice ($n = 6$ per group). **C:** Representative SDF-1 immunofluorescence-labeled proximal tibia sections (green, SDF-1; blue, DAPI); **arrows** indicate selected SDF-1$^+$ stromal osteoblastic cells. Original magnification, ×60. **D:** SDF-1 area/DAPI area. *$P < 0.001$ versus +/+ VEH; †$P < 0.01$ versus +/+ VEH; ‡$P < 0.05$ versus −/− VEH & +/+ PTH. **E:** ELISA analysis of bone marrow SDF-1 levels in 22-week-old $Prg4$ mice, showing marrow SDF-1 (pg/mL) normalized to marrow cell numbers ($n \geq 5$ per group). *$P < 0.05$, versus +/+ VEH; †$P < 0.05$, versus +/+ VEH; ‡$P < 0.05$, versus −/− VEH & +/+ PTH. Data are expressed as means ± SEM.

basal SDF-1 mRNA expression in the marrow. SDF-1 immunofluorescence analysis in proximal tibia (Figure 7, C and D) and SDF-1 ELISA analysis of hindlimb marrow (Figure 7E) demonstrated that SDF-1 protein was reduced in the marrow of $Prg4^{-/-}$ mice, compared with $Prg4^{+/+}$ mice. PTH significantly increased SDF-1 protein expression in the marrow of $Prg4^{-/-}$ and $Prg4^{+/+}$ mice; however, this was blunted in $Prg4^{-/-}$ mice relative to

Prg4^{+/+} littermates ([Figure 7](#), C–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 expression in the bone marrow of the mature skeleton suggests that proteoglycan 4 may affect 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. Considering that more immature osteoblastic cells have been implicated in HPC expansion and function,[43] the finding that basal *Prg4* mRNA expression is higher in less differentiated osteoblastic cell cultures suggests that proteoglycan 4 may be involved in the 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 biological actions in marrow hematopoiesis. Based on 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 to consistently high levels in differentiated osteoblastic cell cultures, despite the lower basal levels, may simply reflect the greater PPR activity in more differentiated osteoblastic cells.[27]

The dysregulated immune cell populations noted in *Prg4* mutant mice may be secondary to the decreased basal expression of marrow SDF-1 protein 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.[39,44–46] Studies have demonstrated that the CXCR4/SDF-1 chemokine axis regulates circulating neutrophil homeostasis.[39,40,47] Injection of CXCR4-blocking antibody in mice mobilized neutrophils from the marrow,[39] and drugs targeting CXCR4 induced a peripheral neutrophilia in mice and humans.[47] Because 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. Although 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 normal physiological conditions, proteoglycan 4 actions supporting B220[+] cell numbers may play a more crucial role during immune response.

The decreased expression of marrow SDF-1 protein 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.[48,49] Mice deficient in SDF-1 have severely reduced B-lymphocyte progenitors in the marrow,[50,51] and studies have demonstrated that SDF-1 supports the homing[40,48,49] and proliferation[52] 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, because IL-6 supports physiological B-lymphopoiesis.[28,53,54] 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,[55] suggesting that osteoblast cell signaling also could play a role in *Prg4* mutant mice. The unexpected finding that PTH induced normalization of peripheral blood neutrophils and marrow B-lymphocytic cells in *Prg4* mutant mice to wild-type 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 and wild-type mice suggests that proteoglycan 4 is not a critical regulator of physiological marrow HPC numbers. The finding that PTH increased Lin[−]Sca-1^{+}c-Kit^{+} cells significantly more in *Prg4* wild-type mice indicates that proteoglycan 4 supports PTH actions on HPC expansion. We speculate that, although basal proteoglycan 4 expression does not affect HPC numbers, the PTH-induced increase in proteoglycan 4 supports HPC expansion, a concept that is supported by HAPO studies.[14,15] Daily intermittent subcutaneous injection of recombinant HAPO in normal mice increases marrow HPC populations, including CD34^{+}, c-Kit^{+}, and Sca-1^{+} cells.[14] Because SDF-1 is a potent chemoattractant that regulates the homing and mobilization of marrow HPC cells,[36,37,56,57] 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.

PTH increased bone marrow and calvarial IL-6 mRNA to a greater extent in *Prg4* mutant mice; thus, in the absence of *Prg4*, PTH differentially regulates the expression of PTH response-associated genes, which may represent an attempt at compensation for the absence of proteoglycan 4. IL-6 supports HPC proliferation,[28,29,58,59] and we have recently shown it to be a critical regulator of PTH actions in HPC expansion.[13]

Because the calvarial organ is composed primarily of stromal 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. Because PTH modulated other responsiveness genes similarly in both *Prg4* mutant and wild-type mice, the heightened IL-6 regulation in *Prg4* mutant mice points to the likelihood of a specific compensatory effect occurring with the loss of proteoglycan 4 activity.

Although 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, similar frequency of marrow CD41^{+} cells, similar marrow megakaryocyte ploidy, and similar number of megakaryocytes per marrow area in *Prg4* mutant and wild-type mice all suggest that proteoglycan 4 is not a critical regulator of physiological 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.[60–64] A single PTH injection significantly increased SDF-1 mRNA in the marrow of wild-type but not mutant mice, which may explain the blunted increase in marrow SDF-1 protein levels in PTH-treated *Prg4* mutant mice. In the light of recent work demonstrating that intermittent PTH administration inhibits dipeptidyl peptidase-IV (DPP-IV),[65] 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.

The present 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 findings from other studies in which proteoglycan 4 was administered, characterization of the *Prg4* mutant mouse model demonstrates that basal proteoglycan 4 expression does not affect megakaryopoiesis or HPC frequency. Although CACP patients have not been reported to spontaneously develop peripheral 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, some of which are compensated for but which could also have implications for immune response. In summary, proteoglycan 4 is a novel immunomodulatory factor regulating physiological immune cell populations and PTH actions on hematopoiesis.

Acknowledgments

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