PTH and Vitamin D Repress DMP1 in Cementoblasts

Journal of Dental Research
2015, Vol. 94(10) 1408–1416
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DOI: 10.1177/0022034515599726
jdr.sagepub.com

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Abstract

A complex feedback mechanism between parathyroid hormone (PTH), 1,25(OH)₂D₃ (1,25D), and fibroblast growth factor 23 (FGF-23) maintains mineral homeostasis, in part by regulating calcium and phosphate absorption/reabsorption. Previously, we showed that 1,25D regulates mineral homeostasis by repressing dentin matrix protein 1 (DMP1) via the vitamin D receptor pathway. Similar to 1,25D, PTH may modulate DMP1, but the underlying mechanism remains unknown. Immortalized murine cementoblasts (OCCM.30), similar to osteoblasts and known to express DMP1, were treated with PTH (1–34). Real-time quantitative polymerase chain reaction (PCR) and Western blot revealed that PTH decreased DMP1 gene transcription (85%) and protein expression (30%), respectively. PTH mediated the downregulation of DMP1 via the cAMP/protein kinase A (PKA) pathway. Immunohistochemistry confirmed the decreased localization of DMP1 in vivo in cellular cementum and alveolar bone of mice treated with a single dose (50 µg/kg) of PTH (1–34). RNA-seq was employed to further identify patterns of gene expression shared by PTH and 1,25D in regulating DMP1, as well as other factors involved in mineral homeostasis. PTH and 1,25D mutually upregulated 36 genes and mutually downregulated 27 genes by ≥2-fold expression ($P \leq 0.05$). Many identified genes were linked with the regulation of bone/tooth homeostasis, cell growth and differentiation, calcium signaling, and DMP1 transcription. Validation of RNA-seq results via PCR array confirmed a similar gene expression pattern in response to PTH and 1,25D treatment. Collectively, these results suggest that PTH and 1,25D share complementary effects in maintaining mineral homeostasis by mutual regulation of genes/proteins associated with calcium and phosphate metabolism while also exerting distinct roles on factors modulating mineral metabolism. Furthermore, PTH may modulate phosphate homeostasis by downregulating DMP1 expression via the cAMP/PKA pathway. Targeting genes/proteins mutually governed by PTH and 1,25D may be a viable approach for designing new therapies for preserving mineralized tissue health.

Keywords: parathyroid hormone, 1,25D, dentin matrix protein 1, mineral homeostasis, RNA sequencing, hormonal regulation

Introduction

A complex feedback mechanism between parathyroid hormone (PTH), 1,25(OH)₂D₃ (1,25D), and fibroblast growth factor 23 (FGF23) maintains mineral homeostasis by regulating calcium and phosphate absorption/reabsorption (Krajisnik et al. 2007; Bergwitz and Jüppner 2010; Lavi-Moshayoff et al. 2010). PTH is the principal hormone that regulates serum calcium concentration by directly acting on the kidneys, bones, and small intestine (Mundy and Guise 1999). PTH targets the kidneys to increase reabsorption of calcium from distal tubules and increase production of 1,25D, which in turn targets the small intestine to enhance calcium and phosphate absorption. Furthermore, by indirectly mediating osteoclastic bone resorption, both PTH and 1,25D enhance calcium release from bones (McSheehy and Chambers 1987; Britto et al. 1994). In turn, the high serum calcium levels activate calcium-sensing receptors located on parathyroid cells to control PTH secretion, thereby creating a negative feedback mechanism to control PTH levels (Tfelt-Hansen and Brown 2005). Independent of the calcium-mediated negative feedback mechanism, an increase in 1,25D concentration represses PTH secretion to regulate PTH levels

(Haussler et al. 2013). Thus, the intricate interaction between calcium, PTH, and 1,25D helps maintain calcium homeostasis.

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A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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FGF23 is the principal endocrine regulator of serum phosphate concentration and is primarily secreted by osteocytes and osteoblasts (Lanske and Razaque 2014). FGF23 acts on the kidneys to increase phosphate excretion and decrease activity of the 25(OH)D-1 α -hydroxylase, which converts 25(OH)D to 1,25D, thereby diminishing intestinal absorption of calcium and phosphate. Both systemic and local bone-derived factors regulate FGF23 promoter activity (Komaba and Fukagawa 2010). FGF23 levels are modulated by phosphate, 1,25D, PTH, and other mineral homeostasis factors, including phosphate regulating endopeptidase homolog, X-linked (PHEX), and the members of the small integrin-binding ligand interacting glycoproteins (SIBLING) family, including dentin matrix protein (DMP1) and matrix extracellular phosphoglycoprotein (MEPE) (Martin et al. 2011).

Previously, we showed that 1,25D regulates FGF23 expression by repressing DMP1 via the VDR pathway (Nociti et al. 2014). It has been hypothesized that similar to 1,25D, PTH may indirectly regulate FGF23 expression by modulating *Dmp1* and/or *Sost* as an intermediary step (Bellido et al. 2005; Gooi et al. 2014). We aimed to define the molecular mechanisms involved in PTH-mediated regulation of *Dmp1*. Furthermore, using RNA sequencing (RNA-seq), we aimed to identify specific genes and signaling pathways mutually regulated by both PTH and 1,25D.

Materials and Methods

Cell Culture and Treatments

Immortalized murine cementoblasts (OCCM.30) were used in cell culture experiments (D'Errico et al. 2000; Nociti et al. 2014). Details of cell culture and PTH and 1,25D treatments are described in the Supplementary Materials and Methods in the Appendix.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

For gene expression analyses, total RNA was isolated using the Qiagen RNeasy Micro kit (Valencia, CA, USA). Complementary DNA (cDNA) synthesis and quantitative polymerase chain reaction were performed on the Roche Lightcycler 480 II System (Roche Applied Science, Indianapolis, IN, USA) using intron-spanning primers (Nociti et al. 2014). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene for the internal control.

SDS-PAGE and Western Blot Analysis

OCCM.30 cells were treated with PTH (1–34) under serum-free conditions for 48 h (Nociti et al. 2014). Rabbit anti-DMP1 C-terminus antibodies (Huang et al. 2008) and IRDye secondary antibodies (LI-COR, Lincoln, NE, USA) were used to detect DMP1 in Western blot. An imaging system (ODYSSEY CLx; LI-COR) with Image Studio Software (LI-COR) was used to quantify protein levels.

Immunofluorescence

OCCM.30 cells were treated with PTH (1–34) for 48 h, then fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 min and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. The cells were blocked in 1% bovine serum albumin (BSA) with 0.3 M glycine (Sigma-Aldrich) and incubated with rabbit anti-DMP1 C-terminus antibodies for 1 h at room temperature. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify staining.

Immunohistochemistry

Animal studies were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA). Mandibles were collected from mice treated with a once-daily subcutaneous injection of 50 μ g/kg PTH (1–34) ($n = 5$) or vehicle ($n = 3$) at age 16 wk for 6 wk (Novince et al. 2012). Decalcified samples were embedded in paraffin and 5- μ m serial sections were prepared for immunohistochemistry using primary antibody against the DMP1 C-terminus. Antibody detection was performed using DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin.

RNA-Sequencing (RNA-seq)

Total RNA was extracted as described above, and RNA integrity was determined with the Bioanalyzer 2100 using the RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA, USA). RNA-seq methods are described in detail in the Supplementary Materials and Methods in the Appendix.

Statistical Analysis

Intergroup differences were evaluated by 1-way analysis of variance (ANOVA) followed by the post hoc Tukey test or by a Student's *t* test (Prism; GraphPad Software, La Jolla, CA, USA).

Results

PTH Downregulates DMP1 Expression in Cementoblasts

PTH (1–34) at 10^{-7} M significantly downregulated (86%) *Dmp1* messenger RNA (mRNA) expression at 3 h in OCCM.30 cells (Fig. 1A). The PTH/PTHrP receptor antagonist, PTH (7–34), had no effect on *Dmp1* mRNA. The inhibitory effect of PTH on *Dmp1* expression was time dependent, with the most consistent potent effect noted at 3 h following treatment (Fig. 1B). Western blot analysis confirmed a 33% decrease of DMP1 protein in cells treated with PTH (1–34) for 48 h (Fig. 1C). Cell numbers over time (48 h) were not affected by PTH treatment (Fig. 1D). Furthermore, PTH (1–34) at 10^{-7} M downregulated *Dmp1* in osteocyte-like MLO-A5 cells (data not shown) similar to 1,25D

Figure 1. Parathyroid hormone (PTH) downregulates *Dmp1* via cAMP/protein kinase A (PKA) signaling in cementoblasts. (A) PTH (1–34) significantly downregulated *Dmp1* messenger RNA (mRNA) expression by 86% in OCCM.30, while the antagonist PTH (7–34) expression had no effect. (B) PTH (1–34) (10^{-7} M) significantly down-regulates *Dmp1* mRNA expression at 3 h and 12 h, with potent effect noted at 3 h. (C) Western blot demonstrates significant reduction in dentin matrix protein I (DMP1) by 33% in the total cell lysate of OCCM.30 harvested 48 h after PTH (1–34) (10^{-7} M) treatment. (D) Cell enumeration assays showed that OCCM.30 cell numbers over time were not affected by PTH (1–34) (10^{-7} M) treatment. (E) Pretreatment of OCCM.30 with transcription inhibitor actinomycin D (AD) (5 μ g/mL) revealed that PTH (1–34) (10^{-7} M) did not affect *Dmp1* mRNA stability, suggesting a direct effect at the transcriptional level. (F) Forskolin (10 μ M), a PKA activator, had similar effects as PTH (1–34), decreasing *Dmp1* expression by 94%, while the protein kinase C (PKC) activator phorbol dibutyrate increased *Dmp1* expression by ~5-fold. (G) PKA inhibitor H-89 (10 μ M) completely inhibited PTH (1–34)-mediated downregulation of *Dmp1* in OCCM.30. (H) cAMP levels were elevated in PTH (1–34) and forskolin-treated cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ as calculated by 1-way analysis of variance.

(Nociti et al. 2014), confirming the ability of both 1,25 and PTH to downregulate *Dmp1* in “cyte”-like cells.

cAMP/PKA Signaling Is Required for PTH-Mediated Downregulation of *Dmp1* in Cementoblasts

PTH treatment of OCCM.30 cells pretreated with actinomycin D did not accelerate the degradation of *Dmp1* mRNA (Fig. 1E), indicating that PTH effects on *Dmp1* were at the transcription level. Treatment of OCCM.30 cells with the protein kinase A (PKA) agonist, forskolin, had effects similar to PTH, while the protein kinase C (PKC) agonist, phorbol dibutyrate, enhanced *Dmp1* mRNA levels (Fig. 1F), suggesting an effect of PKC on basal *Dmp1* levels. In addition, the PKA antagonist H-89 blocked the PTH-mediated downregulation of *Dmp1* (Fig. 1G). The increase in cAMP levels observed in both PTH and forskolin treatments (Fig. 1H) confirmed that the cAMP/PKA pathway was the primary mechanism involved in PTH-mediated downregulation of *Dmp1*.

PTH Reduces DMP1 Protein Localization in Cementoblasts and Dentoalveolar Tissues

Previously, our group and others have published that intermittent PTH administration in rats increases bone formation in the mandible (Hunziker et al. 2000; Kuroshima et al. 2013). The mice used in this study displayed anabolic actions of PTH based on histomorphometric measures as well as serum biochemical data (Novince et al. 2012). Immunofluorescence staining showed that the DMP1 C-terminal peptide was localized in both the nucleus and cytoplasm of vehicle-treated OCCM.30 cells (Fig. 2A, B). Treatment of cells with PTH (1–34) for 48 h resulted in a significant decrease ($P < 0.05$) in DMP1 C-terminal peptide localization in the nucleus (18%) and cytoplasm (38%) (Fig. 2C, D). Decreased nuclear expression of DMP1 in cementoblasts treated with PTH indicates a potential regulatory function of DMP1 in the nucleus of cells

from mineralized tissue that needs to be further investigated. Furthermore, immunohistochemistry revealed decreased localization of DMP1 C-terminal peptide in matrices of alveolar bone, odontoblasts, and cellular cementum of PTH (1–34)-treated mice (Fig. 2E–H).

RNA-seq Analysis of Patterns of Gene Expression Shared by PTH and 1,25D

Using RNA-seq analysis, we identified patterns of gene expression shared by PTH and 1,25D in regulating calcium and phosphate homeostasis, including those associated with cementogenesis. A total of 24,700 genes were differentially expressed in the PTH treatment group, including 477 genes significantly upregulated and 316 genes significantly downregulated ≥ 2 -fold ($P < 0.05$) (Figure 3). A total of 24,273 genes were differentially expressed in the 1,25D treatment group, including 387 genes significantly upregulated and 265 genes significantly downregulated ≥ 2 -fold ($P < 0.05$).

Genes mutually regulated by PTH and 1,25D. PTH and 1,25D mutually upregulated 36 genes and downregulated 27 genes ≥ 2 -fold ($P < 0.05$). Functional categories with subsets of representative genes altered by both PTH and 1,25D are outlined in Figure 3A with a complete gene list in the Table. In addition, PTH and 1,25D inversely regulated 39 genes; that is, 18 genes upregulated by PTH were downregulated by 1,25D, and 21 genes downregulated by PTH were upregulated by 1,25D (Fig. 3B). Ingenuity pathway analysis of genes significantly upregulated and downregulated by both PTH and 1,25D were categorized as hits into functional categories associated with bone and teeth homeostasis, cell growth and differentiation, extracellular matrix regulation, calcium signaling and binding, and transcription factors that potentially modulate *Dmp1* expression (Fig. 3A). We also identified gene categories involving growth of connective tissue and vascular development, indicating that PTH and 1,25D may be involved in novel functional aspects of skeletal biology or in paracrine regulation of other cell types. In addition, some of the key markers for

mineralized tissue such as bone sialoprotein (*Bsp*), osterix (*Osx*), and bone γ -carboxyglutamate protein (*Bglap*) were also identified by RNA-seq.

Figure 2. Parathyroid hormone (PTH) (1–34) reduces dentin matrix protein I (DMP1) levels in mineralized tissue in situ and in cementoblasts in vitro. (A–D) OCCM.30 were treated with 10^{-7} M PTH or vehicle for 48 h. Compared with the nuclear and cytoplasmic localization of DMP1 C-terminal peptide (green) in controls (A), PTH (1–34) treatment for 48 h decreased DMP1 C-terminal peptide in both the nucleus and cytoplasm (B). The nucleus area was circled by blue lines marked by DAPI staining (not shown). DMP1 C-terminal peptides were downregulated by 18% in the nucleus (C) and by 38% in the cytoplasm (D) of OCCM.30, confirming that PTH (1–34) treatment significantly decreased DMP1 protein expression in both subcellular compartments. (E–H) Immunohistochemistry (IHC) staining of mandible sections from control ($n = 3$) or PTH (1–34)-treated mice ($n = 5$) revealed that PTH (1–34) decreased DMP1 localization in matrices of both cellular cementum and alveolar bone (indicated by yellow arrows). Bars indicate 100 μ m in panels A and B. DEN, dentin; CC, cellular cementum; PDL, periodontal ligament; AB, alveolar bone. *** $P < 0.001$, **** $P < 0.0001$ as calculated by the Student's *t* test.

Figure 3. Parathyroid hormone (PTH) and 1,25(OH)₂D₃ (1,25D) regulate extensive target genes in cementoblasts. **(A)** Selected genes mutually regulated by both PTH and 1,25D were manually grouped by category and delineated based on their degree of regulation (red, upregulated; green, downregulated). **(B)** Venn diagram of PTH and 1,25D upregulated (red), downregulated (green), and inversely regulated genes.

Both PTH and 1,25D regulated several genes encoding components of signaling pathways, including Wnt/ β -catenin, BMP, NF- κ B, VDR/RXR activation, and calcium signaling. In addition, PTH activated the PKA signaling pathway, considered to have anabolic effects on bone (Siddappa et al. 2008). These gene lists and extended results can be found in Supplementary Tables 2 and 4, available online. Genes encoding specific transcription factors regulated by PTH and 1,25D are listed in Supplementary Tables 3 and 5, among which WT1, VDR, and Mef2C were mutually regulated by both PTH and 1,25D. Based on data analysis using Genomatix (Ann Arbor, MI, USA), WT1 (binding sequence: tagtggg CGGGagacatgg), VDR (binding sequence: cagcGAAGttctcg aagaggg at ct), and

Mef2C (binding sequence: ttgctttC-TAAatataatcttt), all have potential binding sites on the *Dmp1* promoter.

We also noted that both PTH and 1,25D upregulated proteoglycan 4 (*Prg4*) by 9-fold and 197-fold, respectively. These results are consistent with our previous study that shows PTH enhances endochondral bone formation and the attainment of peak trabecular bone mass by upregulating *Prg4* mRNA expression in bone and liver (Novince et al. 2012). RNA-seq results from the present study suggest that 1,25D may also regulate *Prg4*, contributing to skeletal/cementum homeostasis.

We also explored endogenous factors important for phosphate regulation. PTH downregulated expression of *Dmp1* (5-fold), *Sost* (4-fold), and *Mepe* (4-fold) in OCCM.30, but only *Dmp1* downregulation was statistically significant ($P < 0.05$) (Table). Confirming our previous study using real-time quantitative polymerase chain reaction (RT-qPCR) (Nociti et al. 2014), 1,25D treatment significantly downregulated *Dmp1* (3-fold) and *Phex* (2-fold) and significantly upregulated *Fgf23* mRNA (48-fold).

Validation of RNA-seq by PCR Array

We validated RNA-seq results using the PCR array for a panel of 39 genes. PCR results confirmed transcriptional changes similar to RNA-seq, including genes coregulated in the same direction (e.g., *Dmp1*, *Dspp*, *Vdr*, *Tnfrsf11*, and *Spp1*) and those inversely regulated by PTH and 1,25D (e.g., *Runx2*, *Fgf2*, and *Hdac7*). Transcription factors such as *Mef2c* and *Wt1* implicated in regulation of *Dmp1* expression were also vali-

dated by PCR. A subset of representative genes validated by PCR array is shown in Figure 4, with a complete gene list provided in Supplementary Table 6. Notably, most of the genes in Supplementary Table 6 were consistent with RNA-seq results, with 85% matching in the PTH-treated group and 90% matching in the 1,25D group. However, PCR array results for *Fgf23*, *Phex*, and *Sost* were inconsistent with RNA-seq results.

Discussion

Mineral homeostasis is maintained by a complex feedback mechanism driven by PTH, 1,25D, and FGF23, but many molecular links between these factors remain unidentified and

Table. Genes Mutually Regulated by Parathyroid Hormone (PTH) and 1,25(OH)₂D₃ (1,25D).

Gene Symbol	Gene Name	Type	PTH Fold Change ^a	1,25D Fold Change ^a
Adam11	ADAM metallopeptidase domain 11	Peptidase	+2.59	+3.00
Csf2rb	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	Transmembrane receptor	+5.50	+7.16
Csf2rb2	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	Transmembrane receptor	+3.43	+14.43
Dnmt3b	DNA (cytosine-5)-methyltransferase 3 beta	Enzyme	+2.03	+12.58
E330013P04Rik	RIKEN cDNA E330013P04 gene	Other	+2.67	+2.59
Fgl2	Fibrinogen-like 2	Peptidase	+2.34	+11.96
Fmo1	Flavin containing monooxygenase 1	Enzyme	+5.20	+12.89
Fst	Follistatin	Other	+2.58	+11.98
Gfra1	GDNF family receptor alpha 1	Transmembrane receptor	+7.90	+4.32
Glyat	Glycine-N-acyltransferase	Enzyme	+5.66	+38.00
Gm10445	Predicted gene 10445	Other	+15.78	+46.18
Gzmb	Granzyme B	Peptidase	+33.72	+6.83
Gzmc	Granzyme C	Peptidase	+10.93	+2.31
Igfbp5	Insulin-like growth factor binding protein 5	Other	+6.72	+22.30
Il2rb	Interleukin 2 receptor, beta	Transmembrane receptor	+5.23	+2.73
Mfap3l	Microfibrillar-associated protein 3-like	Other	+4.51	+4.68
Mmp13	Matrix metallopeptidase 13 (collagenase 3)	Peptidase	+4.83	+3.16
Nrp1	Neuropilin 1	Transmembrane receptor	+3.33	+2.08
Osr2	Odd-skipped related 2 (<i>Drosophila</i>)	Transcription regulator	+2.71	+4.69
Parm1	Prostate androgen-regulated mucin-like protein 1	Other	+5.89	+2.87
Pls1	Plastin 1	Other	+2.01	+3.76
Prg4	Proteoglycan 4	Other	+8.82	+196.62
Prrg4	Proline rich Gla (G-carboxyglutamic acid) 4	Other	+17.12	+125.72
Rad52	RAD52 homolog (<i>Saccharomyces cerevisiae</i>)	Other	+2.79	+2.23
Slc25a19	Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	Transporter	+4.96	+6.02
Slc37a2	Solute carrier family 37 (glycerol-3-phosphate transporter), member 2	Transporter	+4.01	+3.31
Slc1a6	Solute carrier organic anion transporter family, member 1a6	Transporter	+4.64	+7.27
Slpi	Secretory leukocyte peptidase inhibitor	Other	+3.15	+81.53
Spata31d1b	Family with sequence similarity 75, member D1	Other	+7.58	+45.21
Tnfrsf11/RANKL	Tumor necrosis factor (ligand) superfamily, member 11	Cytokine	+8.39	+4.93
Tsnaxip1	Translin-associated factor X interacting protein 1	Other	+2.00	+2.58
Ttc39b	Tetratricopeptide repeat domain 39B	Other	+2.07	+2.00
Usp2	Ubiquitin specific peptidase 2	Peptidase	+3.50	+2.26
Vdr	Vitamin D (1,25-dihydroxyvitamin D3) receptor	Nuclear receptor	+8.23	+6.54
Wdr72	WD repeat domain 72	Other	+46.48	+32.63
Wt1	Wilms tumor 1	Transcription regulator	+21.83	+38.61
4833403115Rik	Laeverin	Peptidase	-2.85	-2.34
Adams15	ADAM metallopeptidase with thrombospondin type 1 motif, 15	Peptidase	-6.75	-33.15
Aldh1a3	Aldehyde dehydrogenase 1 family, member A3	Enzyme	-2.23	-2.03
Astn1	Astrotactin 1	Other	-2.81	-2.76
Cacna1i	Calcium channel, voltage-dependent, T type, alpha 1I subunit	Ion channel	-2.44	-2.48
Ces1g	Carboxylesterase 1G	Enzyme	-2.06	-2.52
Cspg4	Chondroitin sulfate proteoglycan 4	Other	-2.21	-2.58
Dmp1	Dentin matrix acidic phosphoprotein 1	Other	-5.42	-2.52
Fam198b	Family with sequence similarity 198, member B	Other	-2.57	-3.39
Gcnt2	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme (I blood group)	Enzyme	-8.11	-2.35
Grhl3	Grainyhead-like 3 (<i>Drosophila</i>)	Other	-2.18	-10.21
Kcnf1	Potassium voltage-gated channel, subfamily F, member 1	Ion channel	-2.94	-2.44
Macc1	Metastasis associated in colon cancer 1	Other	-3.08	-2.70
Mef2c	Myocyte enhancer factor 2C	Transcription regulator	-2.21	-2.49
Odf3l1	Outer dense fiber of sperm tails 3-like 1	Other	-2.07	-26.91
Olfir874	Olfactory receptor, family 8, subfamily B, member 12	G protein-coupled receptor	-3.12	-47.69
Ptger4	Prostaglandin E receptor 4 (subtype EP4)	G protein-coupled receptor	-2.23	-2.59
Rab11fip4	RAB11 family interacting protein 4 (class II)	Other	-6.48	-2.40
Scml4	Sex comb on midleg-like 4 (<i>Drosophila</i>)	Other	-3.02	-3.05
Smpd3	Sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	Enzyme	-3.46	-6.11
Spns2	Spinster homolog 2 (<i>Drosophila</i>)	Other	-2.18	-2.79
Spry4	Sprouty homolog 4 (<i>Drosophila</i>)	Other	-2.16	-2.41
Tbx2	T-box 2	Transcription regulator	-3.16	-3.30
Tnfrsf11b/OPG	Tumor necrosis factor receptor superfamily, member 11b	Transmembrane receptor	-2.28	-2.42
Tpm1	Tropomyosin 1 (alpha)	Other	-2.03	-2.39
Usp43	Ubiquitin specific peptidase 43	Peptidase	-2.40	-2.54
Xaf1	XIAP associated factor 1	Other	-2.61	-2.28

^a+ represents upregulation and - represents downregulation ($P < 0.05$).

Figure 4. Custom polymerase chain reaction (PCR) array validates the RNA-seq data and identifies complex responses to parathyroid hormone (PTH) (1–34) or 1,25(OH)₂D₃ (1,25D). Cementoblasts treated with vehicle (control), PTH (1–34) (10^{−7} M) (A), or 1,25D₃ (10 nM) (B) were evaluated for expression of the indicated genes. Samples were analyzed in triplicate with standard deviations. **P* < 0.05, ***P* < 0.01, ****P* < 0.0001, as calculated by the Student's *t* test.

poorly understood (Krajisnik et al. 2007; Lavi-Moshayoff et al. 2010). We demonstrate for the first time that PTH, like 1,25D, downregulates *Dmp1* expression in murine cementoblasts via PKA/cAMP signaling and decreases DMP1 protein in mouse dentoalveolar tissues. Furthermore, through RNA-seq analysis, we identified transcriptomes mutually regulated by PTH and 1,25D that implicate gene networks involved in bone and tooth homeostasis, calcium signaling and binding, and regulation of extracellular matrix. Based on these data, we hypothesize that PTH and 1,25D both repress *Dmp1* as an intermediary step for modulating downstream targets, including FGF23, and also regulate multiple aspects of skeletal and dental development and homeostasis. However, downregulation of *Dmp1* may not be a universal phenomenon in mineralized tissues, and there may be cell-specific differences and additional local modifiers that affect cell response.

Dmp1 Is a Target Gene Regulated by PTH

DMP1 is an extracellular matrix protein expressed in osteocytes, osteoblasts, odontoblasts, and cementoblasts (D'Souza et al. 1997; MacDougall et al. 1998; Feng et al. 2002). DMP1 plays an important role in mineral homeostasis via its direct effect on mineralization and indirect effect on systemic phosphate homeostasis that are not well understood (He and George 2004; Ling et al. 2005). *Dmp1*^{−/−} mice exhibit hypomineralized

bones and teeth as a consequence of hypophosphatemia related to elevated FGF23 levels (Ye et al. 2004; Feng et al. 2006; Zhang et al. 2011).

Because of the profound and pivotal function of DMP1 in regulating FGF23 expression and systemic phosphate metabolism, we aimed to better understand how 1,25D and PTH regulate DMP1 expression. Previously, we reported that 1,25D represses *Dmp1* expression via the VDR pathway and that class I histone deacetylase (HDAC) (1/2/3) is required (Nociti et al. 2014). Here we show that PTH downregulates *Dmp1* expression in cementoblasts via a PKA/cAMP-dependent mechanism and decreases DMP1 protein in alveolar bone and cellular cementum of mice. In agreement with our findings, Bellido et al. (2005) reported that PTH downregulated DMP1 and osteocalcin in osteocytes, both of which are targets of transcription factor RUNX2. *Runx2* mRNA was not significantly altered in the present study, although both PTH and 1,25D significantly regulated expression of transcription factors *Mef2c*, *Wt1*, and *Vdr*, which we identified as potential regulators of *Dmp1* transcription. Leupin et al. (2007)

demonstrated that PTH downregulates *Mef2c* activation by PKA-mediated control of HDAC5 translocation to the nucleus (Wein et al. 2014), where it interacts with MEF2c, while our past studies suggested that the 1,25D-responsive *Dmp1* promoter sequence is located between 2.5 and 4 kb upstream of the transcription start site (Nociti et al. 2014). A search for predicted transcription factor binding sites using Genomatix suggested that WT1 might have a potential binding site in the *Dmp1* promoter region, but this novel in silico finding requires further validation. Other transcription factors known to regulate *Dmp1*, including OSX, KLF4, and RUNX2 (Miyazaki et al. 2008; Lin et al. 2013; Liu et al. 2013), were not significantly regulated in our RNA-seq studies.

DMP1 May Serve as an Intermediary Link for 1,25D and PTH Regulation of FGF23

Interactions between PTH, 1,25D, and FGF23 direct calcium and phosphate mineral homeostasis, but several aspects of these interactions remain poorly understood. Like DMP1, PHEX is an endopeptidase-processing extracellular matrix protein expressed in bones and teeth (Rowe 2012). Loss-of-function mutations in either DMP1 or PHEX can result in increased circulating levels of FGF23, hypophosphatemia, an inappropriately normal level of 1,25D, and inherited rachitic conditions such as autosomal recessive hypophosphatemic rickets (ARHR) and X-linked

hypophosphatemic rickets (XLH) (Rowe 2012). These data suggest that PHEX and DMP1 may act upstream of FGF23, but the underlying mechanisms remain unclear.

Previously, PTH and PTHrP (1–34) have been shown to downregulate PHEX through the activation of the PKA transduction pathway, resulting in increased *Fgf23* expression (Alos and Ecarot 2005). PTH downregulation of DMP1 may represent an additional link between PTH and FGF23, which parallels the ability for 1,25D to downregulate PHEX and DMP1 (Rowe 2012) and provides 2 mechanisms for feed-forward regulation of FGF23.

Genes That Are Mutually Regulated by PTH and 1,25D in Cementoblasts

Beyond the finding that 1,25D and PTH both decrease DMP1, existing data support complementary roles for PTH and 1,25D in modulation of mineral homeostasis. Thus, we also aimed to identify shared patterns of gene expression of PTH and 1,25D using RNA-seq.

On a transcriptome-wide scale, RNA-seq results confirm that both PTH and 1,25D regulate genes known to direct mineral homeostasis, including transcripts associated with skeletal system development, extracellular matrix mineralization, cell-cell adhesion, ion transportation, and vascular development. A subset of genes overlapped significantly between PTH and 1,25D transcriptomes. We identified these as genes cooperatively regulated by PTH and 1,25D and further analyzed this subset for gene networks involved in regulating calcium and phosphate homeostasis. Both PTH and 1,25D coregulated genes involved in signaling pathways such as Wnt/ β -catenin, BMP, NF- κ B, VDR/RXR activation, and calcium signaling. In support of these findings, other studies on osteocytes have noted that both PTH and 1,25D opposed differentiation-mediated changes in gene expression during osteoblast-to-osteocyte transition but reinforced mature osteocyte markers (St. John et al. 2014; St. John et al. 2015). Furthermore, our study revealed that PTH regulated its target genes via the PKA-mediated signaling pathway and also through alternative activation pathways, similar to the findings noted by St. John et al. (2015).

Proteoglycan 4 (Prg4), which plays an important role in joint lubrication and synovial homeostasis, is a principal regulator of skeletal homeostasis and PTH anabolism. Initially, PRG4 was identified as a novel PTH responsive gene in bone marrow stromal cells and was investigated as a potential regulator of immune cells and the action of PTH on hematopoietic progenitor cells. Previously, we showed that Prg4 supports endochondral bone formation and trabecular bone mass in the developing skeleton and indirectly supports skeletal homeostasis and PTH anabolic actions by protecting joint function in the mature skeleton (Novince et al. 2012). A single subcutaneous injection of PTH (1–34) (1 μ g/g) in C57BL6 wild-type mice significantly increased *Prg4* mRNA in calvaria and long bone as well as liver, indicating *Prg4* is a PTH-responsive gene not only in bone but also in liver and that both marrow- and liver-derived *Prg4* are candidate regulators of skeletal remodeling and the

anabolic actions of PTH. In the present study, we noted that both PTH and 1,25D upregulated Prg4 by 9-fold and 197-fold, respectively. These results suggest that 1,25D upregulation of Prg4 may contribute to skeletal/cementum homeostasis, but the underlying mechanism remains to be determined.

Treatments with 1,25D or PTH induce elevated FGF23 expression in cells in vitro, including osteocytes, osteoblasts, and cementoblasts (Lavi-Moshayoff et al. 2010; Nociti et al. 2014). Our RNA-seq analysis revealed that 1,25D upregulated *Fgf23* transcripts significantly at 24 h. The limited effect of PTH on *Fgf23* and *Sost* at 3 h could be attributed in part to 1) the variable influence PTH exerts on cells based on treatment time and duration (e.g., 3 h may be ideal for detecting *Dmp1* changes but not other genes), 2) low basal levels of certain genes preventing detection and/or statistical assessment, and 3) the experimental design (e.g., in our previous study examining effects of 1,25D on DMP1 expression, cells were seeded at $1.0 \times 10^4/\text{cm}^2$ for 5 d prior to addition of 1,25D) (Nociti et al. 2014). In contrast, in the current study, cells were seeded at a higher density ($2.0 \times 10^5/\text{cm}^2$) for 2 d, prior to adding PTH. Both methods generated consistent results in terms of PTH effect on DMP1 expression, although minor differences were observed in expression of other genes such as *Sost* and *Fgf23*. Future studies should explore the possibility of a time-dependent transient regulation of genes by PTH and 1,25D as well as their effects on mineralization in vitro and in vivo. A further in-depth investigation to understand factors, hormones, and pathways controlling calcium and phosphate homeostasis will have a significant impact on the diagnosis and treatment of disorders of bone and mineral metabolism.

Author Contributions

L. Wang, A.B. Tran, F.H. Nociti Jr, C.C. Krieger, K.R. Kantovitz, M.J. Somerman, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; V. Thumbigere-Math, B.L. Foster, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript; C.M. Novince, A.J. Koh, L.K. McCauley, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This research was supported in part by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases (MJS) and the National Institute of Diabetes and Digestive and Kidney Diseases R01DK53904 (LKM). The authors thank Dr. Chunlin Qin (Baylor College of Dentistry) for providing the DMP1 antibody, Gustavo Gutierrez-Cruz and Steve Brooks from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) for RNA-seq analysis, and Mudita Patel (NIAMS) for technical assistance. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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