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Periosteal PTHrP Regulates Cortical Bone Remodeling during Fracture Healing

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Abstract

Parathyroid hormone-related protein (PTHrP) is widely expressed in the fibrous outer layer of the periosteum (PO), and the PTH/PTHrP type I receptor (PTHR1) is expressed in the inner PO cambial layer. The cambial layer gives rise to the PO osteoblasts (OBs) and osteoclasts (OCs) that model/remodel the cortical bone surface during development as well as during fracture healing. PTHrP has been implicated in the regulation of PO modeling during development, but nothing is known as regards a role of PTHrP in this location during fracture healing.

We propose that PTHrP in the fibrous layer of the PO may be a key regulatory factor in remodeling bone formation during fracture repair. We first assessed whether PTHrP expression in the fibrous PO is associated with PO osteoblast induction in the subjacent cambial PO using a tibial fracture model in PTHrP-lacZ mice. Our results revealed that both PTHrP expression and osteoblast induction in PO were induced 3 days post-fracture. We then investigated a potential functional role of PO PTHrP during fracture repair by performing tibial fracture surgery in 10-week-old CD1 control and PTHrP conditional knockout (PTHrP cKO) mice that lack PO PTHrP. We found that callus size and formation as well as woven bone mineralization in PTHrP cKO mice were impaired compared to that in CD1 mice. Concordant with these findings, functional enzyme staining revealed impaired OB formation and OC activity in the cKO mice.

We conclude that deleting PO PTHrP impairs cartilaginous callus formation, maturation and ossification as well as remodeling during fracture healing. These data are the initial genetic evidence suggesting that PO PTHrP may induce osteoblastic activity and regulate fracture healing on the cortical bone surface.

Keywords

Fracture; Periosteum; Cortical bone; Remodeling; PTHrP

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1. Introduction

Parathyroid hormone-related protein (PTHrP) is a member of the small parathyroid hormone (PTH) gene family [1–2]. Though it shares a common receptor with PTH (referred to as PTHR1), the biological specificity of PTH and PTHrP is completely different [3–4]: PTH is a classical peptide hormone, whereas PTHrP acts as an autocrine/paracrine regulatory factor. Both PTHrP and the PTHR1 are widely expressed and PTHrP has been shown to regulate a variety of processes such as mammary development, tooth eruption, the mobilization of skeletal calcium during lactation, and endochondral bone formation. Its role in endochondral bone is particularly well studied. Here, PTHrP and Indian hedgehog (Ihh) function in a classical feedback loop that regulates the rate of the chondrocyte differentiation progress that drives linear bone growth [5]. Recently, PTHrP expression has been identified in the fibrous periosteum (PO) in which it functions to model the cortical surface during development and at sites of fibrous tendon and ligament insertions with the bony cortex [6].

Even though PTHrP is widely expressed in many tissues throughout life, it was hard to detect its expression level until the generation of the PTHrP-lacZ knock-in mouse. The PTHrP-lacZ knock-in mouse is a useful and sensitive system in identifying sites of PTHrP expression as well as the regulation of gene expression in these sites [7]. This is the system that initially revealed PTHrP expression in the fibrous PO, where it appears to regulate modeling via inducing osteoclasts on the cortical surface [7–8]. Both osteoclast induction and cortical modeling fail in a conditional knock-out (cKO) mouse in which PTHrP was deleted in the fibrous PO via the scleraxis gene (Scx-Cre) [9]. It is not known whether PTHrP influences osteoblast induction or function, but the Scx-Cre cKO mouse displays a rather profound increase in endocortical bone formation and thickness; it is not known if PTHrP regulates endochondral osteoblast function in this system directly or via an osteoclast generated product.

Fracture repair involves a series of processes that regenerate and remodel the bone structure after injury [10–14]. The cells that contribute to this process are derived from the underlying cortical bone and the PO, which contains abundant mesenchymal stem cells that can differentiate into bone and cartilage. After fracture, a soft callus forms at the fracture site and is repaired via chondrocytes that are derived from the PO as well as the injured soft tissue at the fracture site (Figure S1). The PO provides bone cells to the hard callus (PO new bone) that will ultimately bridge the fracture. Over several months, the entire site is remodeled into mature cortical bone. There are many signaling pathways that regulate the chondrogenesis, osteogenesis, and osteoclast formation that drive fracture repair, but a possible role of PTHrP in fracture repair has not been previously examined. Here, we used a PO-dependent open transverse tibial fracture model to study PTHrP inducibility in PTHrP-lacZ mice as well as its putative function during fracture repair in a conditional knock-out (cKO) mouse that lacks PO PTHrP [8]. In brief, whereas unstable fracture models heal via an avascular callus that recapitulates the endochondral pathway, when a long bone is fractured by open surgical transection and stabilized with intramedullary needle fixation it heals via mesenchymal stem cell (MSC)-driven osteoblast formation derived from the highly vascular PO [15–17].

It is clear that PTHrP induction of PO osteoclasts mediates the sculpting aspects of PO modeling, but it is unknown if PTHrP regulates osteoblastic induction in the PO or any other site. In this study, we used a fixed tibial fracture procedure to conduct fracture surgeries in CD1 control and PTHrP cKO mice and determined the role of PO PTHrP in cartilaginous callus formation, maturation and ossification during the fracture repair. We generated PTHrP cKO mice by conditionally deleting it using scleraxis targeting (*Scx-Cre*); *Scx* is a basic helix-loop-helix (bHLH) transcription factor that is expressed in the fascia and connective tissue that binds bones and muscle together and to each other. The mice underwent the fixed tibial fracture procedure at 10 weeks of age, with readouts at days 7, 14, 21, and 28 post-fracture. We found that PTHrP and osteoblasts are induced in parallel shortly after fracture and that deletion of PTHrP impairs cartilaginous callus formation, maturation, and ossification during fracture repair. These data are the initial genetic evidence suggesting that PO PTHrP may induce osteoblastic activity and regulate fracture healing on the cortical bone surface.

2. Materials and Methods

We bred *Scx-Cre* mice with *PTHrP^{lacZ/lox}* mice to generate the PTHrP cKO mice with conditionally deleted PTHrP in the PO. The PTHrP-lacZ replacement construct served as the PTHrP-null allele in this system, providing a convenient lacZ marker of PTHrP-expression sites in the PTHrP cKO mouse. Cross-comparison of β -galactosidase (β -gal)-expressing patterns in the PTHrP-lacZ [7] and *Scx-Cre/R26R* mice allowed us to identify those sites in which PTHrP and *Scx* gene expression [18] were concordant and therefore candidate sites of interest in the cKO mouse (Figure S2). Gender- and age-matched CD-1 (wild-type) mice served as controls. All mice were handled according to the United State Department of Agriculture guidelines and with the approval of the Yale University Animal Care and Use Committee.

2.1. Tibial Fracture Model

An open transverse tibial fracture with intramedullary needle fixation was used as the bone fracture model [19]. Ten-week-old male mice were anesthetized with ketamine (60mg/kg) by intraperitoneal injection. The hair covering the operation sites were shaved by an electric hair shaver. The tibial fracture procedure was performed on the right hind limbs under aseptic conditions as follows: 1) a 1.5cm incision was made in the skin on the antero-medial surface using a scalpel; 2) a 25 gauge needle was inserted into the tibia marrow cavity through the medial side of the tibial plateau at the medial side of the patellar ligament to make a pin canal; 3) the needle was removed and the marrow cavity was suitably enlarged; 4) a No. 11 surgical blade was used to transect the mid-shaft of the tibial diaphysis; 5) the 25 gauge needle was reinserted into bone marrow cavity till the narrowest point to simulate fixation, and the pin beyond tibial plateau was cut off by a wire-cutter; 6) the wound was closed with 4.0 nylon sutures, and buprenorphine was administered in drinking water for pain relief for the first three days after the operation. The left tibia was used as a control and was only exposed by incision, and sutured without manipulating the tibia.

2.2. Radiographic and μ CT Analysis

CD1 and PTHrP cKO mice were sacrificed 7, 14, 21, and 28 days post-fracture. X-ray radiography was performed in both antero-posterior and lateral views (n=5 per time point per group) to examine the fracture pattern and the position of the fixation needle as well as the progress of fracture healing by assessment of bridging across cortices (30kV for 8.0s, Faxitron X-ray, Wheeling, IL). Prior to histological processing, PFA-fixed tibia tissues were evaluated by micro-CT using a Scanco μ CT35 scanner (Scanco Medical AG, Switzerland) with 55kVp source. The samples were scanned at an isotropic resolution of 10 μ m. The scanned images from each group were reconstructed at the same thresholds to allow 3-D structural rendering of each sample. The fracture site was analyzed to quantify the amount of callus mineralized volume fraction (BV/TV, %) and connectivity density (Conn.D, 1/mm³) (n=5 per time point per group).

2.3. Histology and Immunohistochemistry

Mice were sacrificed and tibial samples from each group were harvested and prepared for sectioning and analysis. Toluidine blue staining was performed on non-decalcified sections to characterize the basic histological structure at each time point (n=6 per time point per group). Samples were fixed in 4% Paraformaldehyde (PFA) on ice for 2 hours, followed by decalcifying with 7% EDTA (American Bioanalytical, Natick, MA) at 4°C for 21 days. After washing with PBS and adding magnesium (American Bioanalytical, Natick, MA), samples were processed and embedded in paraffin. Paraffin sections were stained by X-gal (5-bromo-4-chloro-2-indolyl- β -D-galacto-pyranoside), tartrate-resistant acid phosphatase (TRAP), or alkaline phosphatase (ALP) as described [6–7] using a heat step to inactivate endogenous galactosidase activity. Immunohistochemistry for receptor activator of NF- κ B ligand (RANKL) used the protocol from the supplier (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described previously [8]. All data were replicated in triplicate unless otherwise indicated. Histomorphometric measurements were performed using ImagePro Plus 4.5 software (Leeds Precision Instruments, Minneapolis, MN, USA). Total TRAP or ALP positive areas were outlined on projected images of each histologic section to determine enzyme activity.

2.4. Laser Capture Microdissection (LCM) and RT-PCR

Six-week-old CD1 and PTHrP cKO tibial tissues were fixed and demineralized followed by embedding in paraffin and preparing 7 μ m sections on PEN membrane slides (Leica). All the samples were freshly prepared and treated with deionized, diethylpyrocarbonate (DEPC) water. We used a Leica LCM system in the Yale Pathology Core Microscopy Lab with a UV laser on a Leica microscope. Freshly sectioned tibia samples were used to capture PO tissues and RNA were harvested via Arcturus Pico Pure RNA kits and purification columns and RNase-free DNase under manufacturers' instruction [20]. The concentration and quality of the RNA was determined by the Yale Genome Center and 100fg-1 μ g transcribed into cDNA using pre-amplified samples via an RT² PreAmp kit under manufacturer's instruction. RT-PCR was performed with β -actin was the endogenous reference gene. (See Supplementary Material for primers used).

2.5. Statistical Analysis

Results of all quantitative assays involving multiple doses and time points were analyzed using one-way ANOVA followed by Dunnett's test. For experiments comparing two groups, unpaired student's t-test was applied. $p < 0.05$ was considered to be a significant difference.

3. Results

3.1. PTHrP induction after fracture

PTHrP is expressed in the fibrous PO and the PTHR1 in the subjacent cambial PO, whereas Scx is highly expressed throughout the PO (Figure S2). To determine whether the PTHrP gene could be induced in the PO during fracture healing, we performed tibial fractures in 10-week-old PTHrP-lacZ mice and sacrificed mice at day 3 (D3), day 5 (D5), and day 7 (D7) ($n=3$ per group). The adequacy and stability of the fracture were confirmed by Faxitron X-rays (Figure S3). The principal goals here were to see if the fracture would induce PTHrP-lacZ in the PO and, if so, if PO OB induction accompanied this. We found abundant X-gal expression in PO mesenchymal stem cells (MSCs) at the fracture site as compared to the control limb, accompanied by abundant ALP-positive PO osteoblasts at the fracture site (Figure 1A–B). This result was seen in D3 samples, and the osteoblasts persisted at the site at D7, whereas the β -gal signal was waning by D7.

3.2. Decreased bone formation in the PTHrP cKO mice

μ CT (Figure 2A–H) revealed that the callus in CD1 and cKO mice formed by D14, continued at D21, and was near complete at D28. Overall, CD1 mice had more effective repair, with larger total mineralized callus volume compared to the corresponding cKO mice. Quantitation by μ CT revealed a significant decrease in mineralized callus volume (BV/TV, %) at the fracture sites in PTHrP cKO mice at days 14, 21, and 28 compared to CD1 controls (Figure 2I). Connectivity density (Conn.D, $1/\text{mm}^3$), which describes the connectivity of the new bone formed at the fracture callus, was also decreased significantly in cKO mice at day 21 and day 28 (Figure 2J).

3.3. Impaired fracture healing in PTHrP cKO mice

Impaired fracture healing in the PTHrP cKO mice was evident at D14. Toluidine blue-staining revealed that at D14 the fracture site in CD1 mice on the medial side of tibia (Figure 3A, arrowhead) formed a large bridge composed mainly of woven bone. In the PTHrP cKO mice the fracture sites (Figure 3B) revealed clearly impaired repair, displaying fewer chondrocytes, smaller callus size, less woven bone formation, and delayed callus mineralization. This pattern continued throughout the period of repair.

3.4. Decreased osteoblastic activity in PTHrP cKO mice

ALP staining, an indicator for osteoblastic activity, revealed that at D7, D14, D21, and D28 post-fracture, the CD1 sections had significantly more ($p < 0.05$) ALP-positivity compared to cKO sections (Figure 4A–D, dark purple; Figure 4G). CD1 tibia (Figure 4E) displayed a larger area of mineralized woven bone with purple positive ALP staining compared to cKO tibia (Figure 4F). The fracture sites in the cKO mice contained large, round, hypertrophic

chondrocytes surrounded by ALP-positive staining, indicating impaired bone formation and mineralization in the cKO mice as compared to that in the CD1 mice. By D21 and D28 post-fracture, ALP positive staining was decreased in both CD1 and cKO mice, but CD1 mice still displayed significantly more staining than cKO mice (Figure S4). Overall, these results were consistent with our micro-CT and histological data, indicating impaired healing in cKO mice compared to CD1 mice.

3.5. Decreased osteoclastic activity in PTHrP cKO mice

TRAP staining was most striking at D14 (Figure 5). The CD1 and cKO mice both displayed massive TRAP staining although the CD1 mice had formed a larger fracture bridge that had more mineralized tissue as well as more TRAP staining (Figure 5C). By D14 post-fracture, the callus in cKO mice was divided into two parts (Figure 5F) – a chondrocyte-filled inner part surrounded by TRAP-positive cells that lay near the cortical bone surface, indicating that they originated from the PO as indicated by dashed lines. The cKO mice had impaired repair with less TRAP staining and fewer hypertrophic chondrocytes. At D21, TRAP-positive staining was decreasing, and at D28, TRAP activity was no longer detectable in CD1 mice and the fracture site replaced by mineralized bone (Figure S5). The quantification of the TRAP-positive staining area (Figure 5G) revealed that there was 68% less ($p<0.05$) TRAP-positive staining in cKO mice as compared to the CD1 mice 7 days post-fracture and about 45% less ($p<0.05$) TRAP staining in cKO mice compared to CD1 mice at 14 and 21 days post-fracture.

To further determine the mechanism of bone fracture healing in the absence of PTHrP, we performed immunohistochemistry (IHC) for receptor activator of nuclear factor-kappaB ligand (RANKL) to assess osteoclast induction. Consistent with TRAP staining results, the RANKL IHC results revealed decreased RANKL activity in cKO mice as compared to CD1 mice (Figure 6).

3.6. Confirmation of PO PTHrP in chondrogenic and osteogenic development

We isolated PO cells in CD1 and PTHrP cKO mice via laser capture microdissection in order to examine chondrogenic and osteoblastic markers during fracture repair. We examined the expression patterns of Indian hedgehog (Ihh), osteoprotegerin (OPG), bone morphogenetic proteins BMP2, BMP4, and transforming growth factor TGF- β 1. Ihh and OPG are chondrocyte-related genes and were used to confirm the PO PTHrP function in chondrogenic development; BMP2, BMP4, and TGF- β 1 are osteoblast-related genes used to assess osteogenic development. Our results revealed a 2.8x increase in Ihh gene expression ($p<0.05$) (Figure 7A) and a 1.6x increase of OPG gene expression ($p<0.05$) (Figure 7B) in PO of PTHrP cKO mice compared to CD1 mice. Our data also showed that the expression levels of BMP2 (47% decrease, $p<0.05$) (Figure 7C), BMP4 (19% decrease, $p<0.05$) (Figure 7D), and TGF- β 1 (40% decrease, $p<0.05$) (Figure 7E) were all decreased in PTHrP cKO mice compared to CD1 mice. These results corroborated the histological findings described above.

4. Discussion

The periosteum (PO) is an important player in fracture healing. During fracture repair, periosteal progenitor cells respond to the trauma and enter the osteogenic and chondrogenic programs. PTHrP has a number of well-described functions in cartilage and bone development. Quite recently, PTHrP was identified in the fibrous layer of the PO and its receptor in the subjacent PO cambial layer [8]. Conditional deletion experiments in mice have shown that PO PTHrP regulates the modeling of PO surfaces during growth and development as well as the modeling and migration of the fibrous insertions site of tendon and ligament insertion sites into PO cortical bone surfaces [6, 8]. It is clear that PTHrP induction of PO osteoclasts mediates the sculpting aspects of PO modeling, but it is unknown if PTHrP regulates osteoblastic induction in the PO or any other site. Here, we explored whether PTHrP might play a regulatory role in fracture healing. We selected a bone fracture model that is dependent on the PO for the bone cells that mediate fracture repair [11]. In the first set of experiments, we used PTHrP-lacZ mice and found that fracture in this system concurrently induces both PTHrP and osteoblasts in mesenchymal cells at the fracture site. In the second group of experiments, we used mice in which PTHrP had been deleted in fibrous PO and found that fracture repair is profoundly compromised in mice with conditional deletion of PTHrP in the PO.

The repair process of fracture is complicated and the anabolic response driven by new bone formation is critical. Current studies suggest that the newly formed bone during fracture repair could arise from multiple tissue and cell origins including bone marrow, PO, local soft tissue and vasculature [21]. The PO, where PTHrP is expressed, contains abundant mesenchymal stem cells and is clearly a potent source for bone and cartilage differentiation during fracture healing. It has been shown that removal of the PO leads to a significantly diminished capacity for fracture healing from clinical experience and in animal models, causing poor fracture callus formation accompanied with mesenchymal cell deficiency [10, 22–24]. However, the molecular signaling that controls the PO response during fracture healing is not well understood [25]. Recent studies have focused on several signaling pathways and their effects on PO cells. A delayed periosteal activation response and a complete absence of bridging callus upon fracture were found in BMP2 conditional knockout mice, suggesting a role of BMP2 in the initiation of fracture healing [26–28]. Fibroblast growth factors such as FGF2, 5, and 6 are markedly up-regulated during early callus formation and maintain high levels of expression throughout the healing process [29]. *Ihh* also plays critical roles in PO-mediated fracture healing. For instance, it has been shown that *Ihh* is highly expressed during callus formation of both non-stabilized and stabilized fractures [30]. Wnt signaling also contributes significantly to fracture healing. However, studies have shown that Wnt-responsive cells appear to be located mainly on the endosteal surface of injured bone, in contrast to the BMP target cells resident in the PO [31], raising the possibility that, during fracture repair, BMP signaling and Wnt signaling act in different compartments within bone. Thus, a Wnt-based therapy may not directly target the PO, but may indirectly enhance PO-mediated bone regeneration.

Here, we attributed the abnormal healing phenotype in the PTHrP cKO mouse to a failure of modeling and remodeling on the periosteal surface as a consequence of failure of PTHrP

induction of callus formation and osteoclasts on this surface. Based on the RT-PCR results from PO cells in PTHrP cKO mice, one possibility of delayed and impaired fracture healing in cKO mice may be due to PTHrP interaction with TGF- β superfamily proteins. For example, loss of PO PTHrP could down-regulate BMP and TGF- β expression during fracture healing. It has been reported that PTH enhances MSC differentiation to the osteoblast lineage by forming a LRP6/PTH1R complex, thus enhancing BMP signaling [32]. Furthermore, Susperregui et al. showed that BMP2 regulates PTHrP by increasing the expression of PTHrP receptor, which enhances osteoblast differentiation from mesenchymal cell precursors [33]. Further, PTHrP/Ihh and BMP signaling interact to coordinate chondrocyte proliferation and differentiation [34].

In summary, during normal fracture healing, osteoclasts and osteoblasts from the PO, as well as the injured soft tissue at the fracture site, repair the injured site. When PTHrP is knocked out in PO layer, osteoclast and osteoblast expression levels are decreased, leading to impaired fracture repair. The regulation of PO membranous bone remodeling during fracture is poorly understood, but biologically and clinically important. Our findings here provide the initial genetic evidence suggesting that PO PTHrP may induce osteoblastic activity and regulate fracture healing on the cortical bone surface. Even though we have not locally administrated PTHrP in fracture site during fracture healing, our data portray PO PTHrP as an appealing candidate to accelerate fracture healing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We investigate a potential functional role of periosteal PTHrP during fracture repair.
- Periosteal PTHrP is a key regulatory factor in bone fracture repair.
- PTHrP and osteoblast expression in the periosteum were induced after fracture.
- Deletion of periosteal PTHrP impaired fracture healing.

Figure 1. The PTHrP gene was induced in mesenchymal cells after fracture

LacZ staining and ALP staining were performed 3 days post-fracture. In both images, PTHrP expression (greenish blue) was induced in the PO layer 3 days after surgery. The dark purple reflects ALP expression 3 days after fracture. Arrowheads identify ALP-positive osteoblasts/mesenchymal cells, and arrows identify PTHrP-expressing mesenchymal cells. (Scale bars = 100µm in A; 50µm in B).

Figure 2. Decreased mineralized callus volume at fracture site in PTHrP cKO mice

Micro-CT analysis (A–H) showed that in cKO mice callus BV/TV (I) was decreased at 14, 21, and 28 days after fracture, and connectivity density (J) was significantly decreased at 21 and 28 days. (* $p < 0.05$).

Figure 3. Impaired fracture repair in PTHrP cKO mice

Toluidine blue staining was performed in CD1 (A) and cKO (B) mice 14 days post-fracture. Fracture sites in CD1 mice were largely composed of mineralized new PO bone (arrowhead) and soft callus, while fracture sites in PTHrP cKO mice had a greater area of soft callus (arrow) and less mineralized bone. (Scale bar = 400 μ m).

Figure 4. ALP staining

ALP staining (dark purple) showed decreased new woven bone formation in cKO mice (**B**, **D**) compared to CD1 mice (**A**, **C**), indicating impaired fracture healing in cKO mice especially at 7 and 14 days post-fracture (**G**). By D14, CD1 mice had a larger area of mineralized woven bone near the PO with positive ALP staining (**E**) compared to the callus in the cKO mice; the fracture site in the cKO mice contained large, round, hypertrophic chondrocytes surrounded by ALP-positive staining (**F**), indicating impaired bone formation and mineralization. (Scale bars = 400 μ m in A–D; 50 μ m in E–F).

Figure 5. Decreased TRAP activity in cKO mice

TRAP staining (red) showed decreased osteoclast activity in cKO mice (**B, D**) as compared to CD1 mice (**A, C**), reflecting impaired fracture healing in the cKO mice (**G**). By day 14, CD1 mice showed clear new bone formation from PO with massive TRAP-positive staining associated with modelling (**E**). In cKO mice, the fracture bridge was filled with chondrocytes (**F**). The dashed line in Figure 5F separates the chondrocyte-filled inner portion and TRAP-positive mineralized cells near the cortical bone surface, indicating that they originated from PO. All four slides (A–D) were stained at the same time with the same solutions. (Scale bars = 400µm in A–D; 50µm in E, F).

Figure 6. Decreased RANKL activity in cKO mice

RANKL IHC at day 14 showed decreased RANKL activity in cKO mice (**B**) as compared to CD1 mice (red staining, **A**). Figure 6C is a magnified image of Figure 6A. The slides were counterstained with Weigert's iron hematoxylin Solution A&B. RANKL expression is reflected by red staining. (Scale bars = 50 μ m in A, B; 25 μ m in C).

Figure 7. Gene expression in PO PTHrP cKO mice

Six-week-old CD1 and PTHrP cKO tibial PO cells were isolated via laser capture microdissection and RT-PCR was performed. RNA expression analysis showed increased *Ihh* and *OPG* expression (A–B) and decreased *BMP2*, *BMP4*, *TGF-β1* expression (C–E) in cKO mice compared to CD1 mice. (* $p < 0.05$).