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Teriparatide (human PTH_{1–34}) Compensates for Impaired Fracture Healing in COX-2 Deficient Mice

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Abstract

Genetic ablation of cyclooxygenase-2 (COX-2) in mice is known to impair fracture healing. To determine if teriparatide (human PTH_{1–34}) can promote healing of *Cox-2*-deficient fractures, we performed detailed *in vivo* analyses using a murine stabilized tibia fracture model. Periosteal progenitor cell proliferation as well as bony callus formation was markedly reduced in *Cox-2*^{−/−} mice at day 10 post-fracture. Remarkably, intermittent PTH_{1–34} administration increased proliferation of periosteal progenitor cells, restored callus formation on day 7, and enhanced bone formation on days 10, 14 and 21 in *Cox-2*-deficient mice. PTH_{1–34} also increased biomechanical torsional properties at days 10 or 14 in all genotypes, consistent with enhanced bony callus formation by radiologic examinations. To determine the effects of intermittent PTH_{1–34} for callus remodeling, TRAP staining was performed. Intermittent PTH_{1–34} treatment increased the number of TRAP positive cells per total callus area on day 21 in *Cox-2*^{−/−} fractures. Taken together, the present findings indicate that intermittent PTH_{1–34} treatment could compensate for COX-2 deficiency and improve impaired fracture healing in *Cox-2*-deficient mice.

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Conflict of interest

All authors have no conflicts of interest.

Keywords

Cyclooxygenase-2 (COX-2); Fracture healing; Parathyroid hormone (PTH); Periosteum; Prostaglandin

1. Introduction

Fracture healing is a sequential process involving inflammation, endochondral and intramembranous callus formation, and callus remodeling. During the inflammatory stage, various growth factors, cytokines, and local hormones are produced or released at the fracture site to stimulate stem cell proliferation, migration, and differentiation. Endochondral bone formation is initiated in the hypoxic avascular region immediately adjacent to the fracture site when mesenchymal progenitor cells undergo chondrogenesis. As these chondrocytes proliferate and mature, they form a mineralized cartilage matrix allowing for vessel invasion and subsequent bone formation. Distal to the fracture site where vascular integrity is preserved, mesenchymal progenitors differentiate directly into osteoblasts initiating intramembranous bone formation. Through both endochondral and intramembranous bone formation, a mineralized callus of woven bone bridges the fracture site. This callus is eventually remodeled reestablishing the original architecture of the cortical bone.

Cyclooxygenases (COXs) are responsible for the generation of prostaglandins, prostacyclins and thromboxanes, collectively known as the prostanoids, during inflammation. COX-2 is highly expressed in chondrocytes and chondroprogenitors during the early stage of fracture repair [1, 2]. Animal studies have shown that genetic ablation of COX-2 and inhibition of COX-2 activity by non-steroidal anti-inflammatory drugs (NSAIDs) lead to perturbation in temporally distal events such as chondrogenesis, bone formation, and remodeling [3–6]. In contrast, local retroviral delivery of COX-2 accelerates fracture healing and bone union [7]. Prostaglandin E2 (PGE2), one of the prostanoids produced by COX-2, is thought to exert its bone anabolic effects through the EP2 and EP4 receptor subtypes via the production of cAMP and subsequent activation protein kinase A (PKA) [8–11]. In fact, an EP2 selective agonist, shown to increase intracellular cAMP levels, induced bone healing in a canine tibial fracture model. Furthermore, an EP4 selective agonist rescued the inefficiencies in chondrogenesis and reduced bone formation observed in COX-2 knockout murine fractures [12]. These findings suggest that defective fracture healing associated with COX-2 deficiency may be due to reduced PKA activation via PGE2.

Teriparatide (recombinant human parathyroid hormone 1–34 (PTH_{1–34}); Forteo (Eli Lilly, Indianapolis, IN)) is derived from the first 34 amino acids of PTH and is approved by the U.S. FDA to treat osteoporosis [13]. PTH has also been shown to promote fracture healing in animal studies [14–18]. Additionally, two clinical trials have been published describing intermittent PTH treatment for fracture repair [19–21]. These studies showed that PTH could accelerate fracture healing in distal radial and pelvic fractures of elderly osteoporotic patients. The PTH receptor (PTH1R), similar to the EP receptors, is a G-coupled 7-transmembrane receptor that activates PKA signaling. Because of the similar signaling

effects of PGE2/EP4 and PTH/PTH1R, we hypothesized that PTH₁₋₃₄ would rescue the defective fracture healing observed in *Cox-2* deficient mice.

In the present study, we investigated the effects of intermittent PTH1–34 therapy for tibial fracture healing of wild type, *Cox-2* heterozygous (*Cox-2*^{+/-}) and *Cox-2* knockout (*Cox-2*^{-/-}) mice. Radiologic, histologic and biomechanical analyses were performed to document the characteristic changes of fracture healing in those genotypes with or without PTH treatment, as well as real-time RT-PCR and immunohistochemical analyses to assess the temporal and spatial patterns of chondrogenesis, osteogenesis, and osteoclastogenesis related to PTH treatment.

2. Materials and Methods

2.1. Experimental animals

All animal studies were done in accordance with the approval of the University of Rochester Committee on Animal Resources. Healthy male and female, 8- to 9-week-old wild type, *Cox-2*^{+/-} and *Cox-2*^{-/-} mice were used in this study. *Cox-2*^{-/-} mice were originally obtained from the breeding colony maintained at the University of North Carolina and intercrossed for about 30 generations to a 129/ola genetic background [2, 22]. In all experiments, wild-type littermates were used as controls for *Cox-2*^{+/-} and *Cox-2*^{-/-} mice. These mice were housed 5 per microisolator cage in a vivarium housing room on a 12-hour light/dark cycle and provided a free access to a regular diet.

2.2. Tibia fracture model

Mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (4 mg/kg). Following sterile preparation of the surgical site, a 4 mm longitudinal incision was made on the anterior side of the right tibia. A small hole was drilled into the tibial tuberosity using a 26-gauge needle. A transverse osteotomy was performed with a No. 11 scalpel blade at the proximal diaphysis of the tibia. The fibula was not broken. The bone fracture was fixed with an intramedullary nailing procedure using a 26-gauge Quincke type spinal needle (BD Medical Systems, Franklin Lakes, NJ). The wound was closed using 5–0 nylon sutures. After surgery, the mice were divided into two treatment groups: PTH₁₋₃₄ (Forteo; 40 µg/kg) and control (normal saline). Daily subcutaneous drug administration was delivered for a maximum of 3 weeks. A Faxitron Cabinet X-ray System (Faxitron X-ray Corporation, Lincolnshire, IL) was used to take X-ray images at the time of surgery and at 7, 10, 14 and 21 days following surgery until sacrifice.

2.3. Micro-computed tomography (micro-CT)

Fractured tibiae were harvested at the indicated time points, fixed, and scanned using a VivaCT 40 (Scanco Medical AG, Bassersdorf, Switzerland) at a 10.5 µm isotropic resolution with an integration time of 300 ms, energy of 55 kVp, current of 145 mA, and 1000 projections over a 180° rotation. The threshold for segmentation was set at 298 mg HA/cm³. The threshold was chosen using 2D evaluation of several slices in the transverse anatomic plane so that mineralized callus was identified, but surrounding soft tissue was excluded. To analyze external callus bone volume, two contours were manually created on key slices; the

first traced the perimeter of the external fracture callus (inclusion contour) and the second surrounded all cortical bone from the adjacent uninjured cortices (exclusion contour). The interpolation (morphing) tool was used to generate contours in between the key slices, which were inspected for accuracy by the operator to ensure encompassing the entire external fracture callus.

2.4. Biomechanical torsion testing

The torsional biomechanical properties of the fractured tibias were determined using an EnduraTec TestBench system (200N.mm torque cell; Bose Corporation, Minnetonka, MN) at a rate of 1°/sec as previously described [23]. Ultimate torque, yield torque, torsional rigidity, and energy to failure at ultimate torque were determined for each specimen.

2.5. Histology and histomorphometric analyses

Mice were sacrificed at 3, 5, 7, 10, 14 and 21 days after fracture. Tibias were disarticulated from the knee and trimmed to remove excess muscle and skin. Specimens were fixed in 10% neutral buffered formalin for 3 days. The tissues were then processed and embedded into paraffin. Alcian blue hematoxylin/orange G eosin or tartrate resistant acid phosphatase (TRAP) staining was performed as previously described [6]. Histomorphometric analysis was performed using OsteoMeasure (Osteometrics, Inc., Decatur, GA) software to determine the area of total fracture callus, bone, cartilage and mesenchyme (a subtraction of total callus from bone and cartilage tissue) in the external fracture callus (4mm length around the fracture site). Osteoclast numbers were determined from TRAP stained sections by counting the number of TRAP-positive cells in the external callus (4mm length around the fracture site). The mean of at least three non-consecutive sections per sample was used for analysis and a minimum of four samples included in each group. Cortical bone and internal callus were excluded from the histomorphometric analyses.

2.6. Immunohistochemistry

Immunohistochemical staining for nuclear antigen PCNA protein was performed using the PCNA staining kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Total cell number and percentage of PCNA positive cells in the periosteal region near the osteotomized site were counted manually. The mean of three non-consecutive sections per sample was used for analysis and three samples were included in each group at each time point.

2.7. Real-time RT-PCR analyses

Fracture calluses (approximately 4mm in length) including cortical bone were carefully dissected free of muscle on days 0, 3, 5, 7, 10, 14 and 21 following surgery. Bone marrow was flushed out using phosphate-buffered saline (PBS), and the samples were immediately snap frozen in a liquid nitrogen bath. Frozen tissue samples were homogenized with QIAzol (Qiagen, Valencia, CA) using the TissueLyser system (Qiagen) and total RNA was extracted from the samples using the RNeasy Lipid Tissue Mini Kit (Qiagen). Exactly 1 µg of total RNA per callus was used in reverse transcription to make single-strand cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen Corp.). Quantitative PCR

reactions were performed using PerfeCTa SYBR Green FastMix (Quanta BioSciences, Inc., Gaithersburg, MD) in a RotorGene real time PCR machine (Corbett Research, Carlsbad, CA). Gene expression was normalized to β -actin. Gene-specific primers are shown in Supplemental Table 1.

2.8. Statistical analysis

Data are presented as mean $\pm$ standard error (SEM). Statistical significance between experimental groups was determined using heteroscedastic t-tests. Normality was confirmed using Shapiro-Wilk tests. For each time-point of each outcome variable, seven comparisons were made: *Cox-2*^{+/-} PTH vs. *Cox-2*^{+/-} control, *Cox-2*^{-/-} PTH vs. *Cox-2*^{-/-} control and wild type (WT) control vs. each of WT PTH, *Cox-2*^{+/-} control, *Cox-2*^{+/-} PTH, *Cox-2*^{-/-} control, and *Cox-2*^{-/-} PTH. The overall Type I error rate across these seven comparisons was controlled using the bootstrap method to adjust the *P* values. Analyses were performed in SAS version 9.3 (Cary, NC), with bootstrap-adjusted *P* values <0.05 considered statistically significant.

3. Results

3.1. PTH₁₋₃₄ enhances bony callus formation in *Cox-2*^{-/-} fractures

It is well established that COX-2 deficiency impairs fracture healing in mice [6]. To determine whether PTH₁₋₃₄ can compensate for the lack of COX-2 during fracture healing, we administered PTH₁₋₃₄ to wild type, *Cox-2*^{+/-} or *Cox-2*^{-/-} mice at a dose of 40 μ g/kg daily for up to three weeks following fracture using a stabilized, tibia osteotomy fracture model as previously published [24]. Continuous radiographs were taken on days 10 and 14 during the fracture healing process. Mineralized external callus was observed in the fractures of wild type and *Cox-2*^{+/-} control mice at day 10, but not yet apparent in the fractures of *Cox-2*^{-/-} mice at this time point (Fig. 1A). Although bone union was seen in all groups at day 14, a radiolucent line still remained in *Cox-2*^{-/-} control fractures. Intermittent PTH₁₋₃₄ treatment radiographically enhanced fracture callus formation in all genotypes, including *Cox-2*^{-/-}, at days 10 and 14 post-fracture.

To further evaluate mineralized callus formation, micro-CT was performed on fractured tibias harvested at days 7, 10, 14 and 21. Consistent with radiographic observations, external bony callus formation was similar between wild type and *Cox-2*^{+/-} control groups, but reduced and delayed at early time points in comparison in fractures from *Cox-2*^{-/-} control mice (Fig. 1B). Quantitative assessment showed that external bony callus volume was significantly decreased in fractures of *Cox-2*^{-/-} control mice compared to wild type control mice at day 10 (> 2-fold, *p* = 0.0045) (Fig. 1C). Intermittent PTH₁₋₃₄ treatment compensated for the impaired bony callus formation in *Cox-2*^{-/-} mice on days 7 and 10, and significantly increased bony callus volume in all three genotypes from days 10 to 21 when compared to control-treated groups. Most of the fracture callus disappeared at 42 days after fracture in all genotypes (data not shown). Bony callus connectivity density had a similar pattern to bony callus volume during fracture healing (Supp. Fig 1A). Bone mineral density (BMD) of the fracture callus, however, was significantly decreased at 14 and 21 days following fracture in all PTH₁₋₃₄-treated groups compared to control-treated groups (Supp.

Fig. 1B). This is consistent with previous reports showing that while PTH₁₋₃₄ enhances bone matrix deposition, it delays matrix mineralization [25]. Collectively, these data indicate that intermittent PTH₁₋₃₄ treatment can compensate for the impaired bony callus formation of *Cox-2*^{-/-} mice during the early stages of fracture repair.

3.2. Increased biomechanical strength of fractured tibiae in PTH₁₋₃₄-treated mice

In order to determine if intermittent PTH₁₋₃₄ treatment accelerates bone union in wild type, *Cox-2*^{+/-} and *Cox-2*^{-/-} fractures, biomechanical torsion testing was performed on fractured tibias at 10, 14, 21 and 42 days. *Cox-2*^{-/-} fractured tibias had a significantly decreased ultimate torque (> 1.75-fold, *p* = 0.0363) and yield torque (> 1.85-fold, *p* = 0.0041) at 10 days after fracture compared to wild type controls (Fig. 2A and 2B). *Cox-2*^{+/-} fractures behaved similarly to wild type fractures. Intermittent PTH₁₋₃₄ treatment increased the ultimate torque of *Cox-2*^{-/-} fractures to levels similar to those of wild type and *Cox-2*^{+/-} controls on day 10. No significant differences were detected among genotypes in the biomechanics of day 14 and day 21 fractures. Interestingly, at day 42, the biomechanical properties of the *Cox-2*^{-/-} fractures had continued to increase. In contrast, fractures in wild type mice had a peak of mechanical properties/strength at 21 days and then had a slight, non-significant, decline by day 42 post-fracture, likely related to remodeling of the callus as previously described by us and others [24, 26]. Thus, while intermittent PTH₁₋₃₄ treatment enhances fracture healing in both wild type and *Cox-2*-deficient mice, differences likely remain in the course of the remodeling process. Most importantly, these results suggest that intermittent PTH₁₋₃₄ treatment improves the delayed fracture healing observed at early time points in *Cox-2*^{-/-} mice.

3.3. PTH₁₋₃₄ improves impaired fracture callus formation in *Cox-2*^{-/-} mice

Histology and quantitative histomorphometric analyses were performed on fractured tibiae harvested at days 5, 7, 10, 14 and 21 to obtain a more detailed picture of the fracture healing process in all groups of mice. Histology revealed a delay in both cartilage and bone callus formation during fracture healing in *Cox-2*-deficient mice compared to wild type and *Cox-2*^{+/-} mice (Fig. 3A and Suppl. Fig. 3). Specifically, bone callus area as determined by histomorphometry was significantly decreased in *Cox-2*^{-/-} control-treated mice at days 7 (> 2.87-fold, *p* = 0.0017), 10 (> 2.12-fold, *p* = 0.0072), and 21 (> 1.56-fold, *p* = 0.0055) compared to wild type control-treated mice (Fig. 3C). Intermittent PTH₁₋₃₄ treatment enhanced callus formation at these time points in all genotypes. These results are consistent with the micro-CT results. Total area measurements reflected bone callus area measurements with significant decreases in *Cox-2*^{-/-} control-treated fractures at days 7 (> 2.29-fold, *p* = 0.0048), 10 (> 1.53-fold, *p* = 0.0383) and 21 (> 1.40-fold, *p* = 0.0084) (Fig. 3B). Again, intermittent PTH₁₋₃₄ treatment was able to rescue callus formation in *Cox-2*^{-/-} mice and significantly enhance callus formation in wild type and *Cox-2*^{+/-} mice. No significant differences in cartilage area or undifferentiated mesenchyme area were found in any genotype (Figs. 3D and 3E), however, intermittent PTH₁₋₃₄ treatment did increase mesenchyme area at day 7 in *Cox-2*^{-/-} fractures.

In addition to histomorphometry, we compared chondrocyte and osteoblast associated gene expressions over time during the healing process. Consistent with the histomorphometry

data, no significant differences in cartilage transcript levels (*Sox9*, *Col2a1* and *Col10a1*) were found among genotypes (Fig. 4). Additionally, *Runx2* and *Sp7* expression levels were not greatly affected by either genotype or PTH₁₋₃₄ treatment during the healing process suggesting that perhaps commitment of progenitor cells to the osteoblast lineage is unaffected by COX-2 and PTH signaling. *Bglap* expression, however, was significantly reduced in *Cox-2*^{-/-} fractures at day 10 (> 3.60-fold, *p* = 0.0211), but rescued by PTH₁₋₃₄ treatment suggesting that COX-2 is required for osteoblast maturation and that PTH is sufficient to induce maturation in settings of *Cox-2* deficiency. Collectively, these results support that intermittent PTH₁₋₃₄ treatment improves bony callus formation in *Cox-2* deficient mice, and enhances intramembranous bone formation at later stages in all genotypes.

3.4. PTH₁₋₃₄ increases the proliferation of periosteal progenitor cells during the early stages of fracture healing

Cellular proliferation in the external callus from wild type, *Cox-2*^{+/-} and *Cox-2*^{-/-} mice was examined histologically at post-fracture days 3, 5, and 7 via the quantification of PCNA-positive cells (Fig. 5A). At 7 days after fracture, the percentage of PCNA-positive cells within the periosteum surrounding the fracture site was significantly less for both *Cox-2*^{+/-} (> 3.59-fold, *p* = 0.0023) and *Cox-2*^{-/-} (> 3.82-fold, *p* = 0.0135) control-treated mice compared to that for wild type control mice (Fig. 5B). Total cell numbers in the periosteal region were also significantly reduced for both *Cox-2*^{+/-} (> 1.62-fold, *p* = 0.0310) and *Cox-2*^{-/-} (> 2.35-fold, *p* = 0.0074) control-treated mice on day 7 compared to wild type control mice (Fig. 5C). PTH₁₋₃₄ treatment rescued proliferation in *Cox-2*^{+/-} and *Cox-2*^{-/-} mice at day 7 and enhanced proliferation of this cellular population in wild type mice. Total cell numbers in the external callus were increased with PTH treatment in wild type and *Cox-2*^{+/-} mice at day 5 and in all genotypes by day 7. Real-time RT-PCR showed that *Ccnd1* expression, a marker of proliferating cells and a target gene of PTH/PTH1r signaling, was significantly increased by PTH₁₋₃₄ treatment at 3 days after fracture in all genotypes (Fig. 5D). These data are consistent with the mesenchyme area data (Fig. 3E) determined by histomorphometric analyses and suggest that intermittent PTH₁₋₃₄ treatment enhances proliferation of periosteal progenitor cells during the early stages of fracture healing.

3.5. Osteoclast numbers and function are modestly affected by Cox-2 deletion and PTH₁₋₃₄ treatment during fracture healing

To determine the effects of *Cox-2* deletion and PTH₁₋₃₄ treatment on fracture callus remodeling, TRAP staining was performed to quantify osteoclast numbers (Fig. 6A). The number of TRAP-positive cells per total callus area was significantly less in *Cox-2*^{-/-} control-treated mice compared to wild type control-treated mice at days 7 (> 2.17-fold, *p* = 0.0454) and 10 (> 2.92-fold, *p* = 0.0047) post-fracture (Fig. 6B). PTH₁₋₃₄ treatment was unable to rescue osteoclast numbers at these time points. At 21 days post-fracture, however, PTH₁₋₃₄ administration significantly enhanced osteoclast numbers in *Cox-2*^{-/-} mice. Interestingly, PTH₁₋₃₄ did not affect osteoclast numbers in wild type or *Cox-2*^{+/-} mice. Real-time RT-PCR revealed that PTH₁₋₃₄ significantly increased *Trap* expression at day 7 in *Cox-2*^{-/-} mice and at day 21 in wild type mice (Fig. 6C).

4. Discussion

Our previous studies showed that COX-2 was indispensable for a cascade of events that lead to efficient endochondral ossification and bone union during the fracture repair process [2, 6, 12]. Similarly, the current mouse tibia fracture model demonstrated that total and bony callus formation was markedly reduced by *Cox-2* deletion. The metabolites of COX-2 have been shown to induce strong effects on bone metabolism. Among them, PGE2 has potential anabolic effects on osteoblast proliferation and differentiation, and bone formation in animal models and in humans [27–30]. Furthermore, selective prostaglandin EP2 and EP4 receptor agonists have been shown to enhance fracture healing in animal models [9, 31–33].

Here, similar anabolic effects on fracture healing were obtained using intermittent PTH_{1–34} treatment in wild type, *Cox-2*^{+/-} and *Cox-2*^{-/-} fractures. PTH_{1–34} could rescue defective bony callus formation in COX-2 mutant mice. PTH_{1–34} also increased the early biomechanical torsional properties in *Cox-2* deficient fractures. PTH is a small polypeptide hormone that binds to a G protein-coupled receptor and signals to the nucleus through a cyclic AMP-dependent PKA and PKC pathway, similar to the COX-2-PGE2-EP4 signaling pathway. PTH has effects on both bone formation and resorption with chronic elevations in serum PTH concentration predominantly stimulating bone resorption. In contrast, intermittent administration leads to transient increases in serum PTH concentration and a predominantly osteogenic effect. Systemic administration of PTH_{1–34} accelerates the restoration of bone mechanical properties following fracture by augmenting osteogenesis at fracture sites [14, 15, 17, 18, 34, 35]. Although less is known about the role of PTH in cartilage metabolism, signaling through the PTH/PTHrP receptor (PTH1r) in the growth plate has been shown to be essential in chondrocyte differentiation and maturation during skeletal growth [36, 37]. While the effects of intermittent PTH_{1–34} treatment on cartilaginous callus formation did not reach significance in this study, there was a trend towards increased cartilage area in response to PTH_{1–34} at early and mid-stage repair regardless of genotype. PTH and PTHrP were shown to stimulate both proliferation and the synthesis of proteoglycans in fetal chondrocytes but not their differentiation [38–42]. Activation of PTH1R also induced chondrocyte proliferation and matrix production while suppressing maturation. Similarly, intermittent PTH_{1–34} treatment inhibited articular chondrocyte maturation in an osteoarthritis mouse model *in vivo* [43]. Therefore, one effect of PTH during fracture repair might be to increase periosteal cell proliferation, including immature chondrocytes, while inhibiting their maturation.

Nakajima et al. demonstrated that both the number of PCNA-positive subperiosteal osteoprogenitor cells and osteocalcin (*Bglap*) expression (the marker of mature osteoblasts) was significantly increased in the calluses of rat PTH-treated fractures and concluded that treatment of fractures with intermittent PTH administration enhances callus formation by stimulation of proliferation and differentiation of osteoprogenitor cells, thereby, increasing production of bone matrix proteins [35]. In the current study, we observe decreased periosteal progenitor cell proliferation in COX-2 mutant mice (*Cox-2*^{+/-} and *Cox-2*^{-/-}) and increased periosteal progenitor cell proliferation in response to intermittent PTH_{1–34} treatment in fracture calluses of all genotypes examined. PTH_{1–34} treatment was also able to rescue *Bglap* expression in *Cox-2*^{-/-} fractures at day 10. Collectively, these data suggest that

intermittent PTH₁₋₃₄ treatment is able to compensate for loss of COX-2 in fracture healing by promoting periosteal progenitor cell proliferation and subsequent osteoprogenitor differentiation resulting in improved bony callus formation in the *Cox-2*^{-/-} fractures. Whether PTH₁₋₃₄ directly affected osteoblast maturation in our model is unclear. Only in the context of *Cox-2* deficiency did PTH₁₋₃₄ treatment result in significantly increased expression of osteoblast maturation factor *Bglap*. If this is due to enhanced osteoblast maturation or simply increased numbers of committed osteoblasts subsequent to progenitor cell expansion warrants further investigation. Interestingly, lower callus mineral density was observed in the PTH₁₋₃₄-treated fracture calluses. Callus mineral content, however, was higher following PTH₁₋₃₄ treatment given the magnitude of increase in the total callus volume of these fractures (data not shown). This suggests that bone matrix deposition is occurring faster than matrix mineralization in the PTH₁₋₃₄-treated fractures. This may, again, be due to PTH₁₋₃₄-induced proliferation of periosteal progenitor cells. An increase in cell number would provide a larger callus and more bone matrix leading to stronger biomechanical properties even if callus mineralization is delayed. Previous studies have indicated that PTH₁₋₃₄ stimulates the proliferation of immature osteoblasts, but inhibits differentiation of more mature cells [44–46]. In contrast, some papers could not provide the evidence of increased cell proliferation by PTH₁₋₃₄, but suggested that intermittent PTH₁₋₃₄ treatment enhanced the differentiation of osteoblasts [47–50]. This inconsistency may be due to the differences in the differentiation stage of osteoblastic cells at the time of PTH₁₋₃₄ treatment.

The increase in bony callus formation was accompanied by increased osteoclast number at 3 weeks post-fracture. Exogenous PTH₁₋₃₄, therefore, not only stimulated bony callus formation by enhancing osteoblastic bone formation but also accelerated bony callus remodeling by stimulating osteoclastic bone resorption. Osteoclasts rise from mononuclear precursors in the bone marrow space via the interaction with osteoblasts or stromal cells, which provide a milieu of osteoclastogenic cytokines (mainly RANKL and M-CSF) required for the differentiation process. Stimulated osteoclast formation in cultures is frequently found to be prostaglandin-dependent [51, 52]. The suggestion that decreased total prostaglandin production limits the amount of bone resorption is supported by the fact that NSAIDs have been reported to increase BMD in both animal and human studies [53–56]. These data suggest that PTH₁₋₃₄ also could rescue an impaired callus remodeling by increased osteoclast formation.

NSAIDs such as COX-2 inhibitors are clinically used for pain control after fracture. Some animal experiments showed that COX-2 inhibitors reduced bone repair to the same degree as that in *Cox-2*-deficient mice [5, 57, 58]. We previously showed that, with aging, reduced COX-2 callus expression accompanies a decreased rate of fracture healing [1]. Using the same stabilized tibia osteotomy model as that described here, we also showed that PTH₁₋₃₄ treatment could enhance progenitor cell proliferation and bony callus formation in fractures of aged mice [24]. Remarkably, the aged fractures also had higher biomechanical properties at day 42 which were enhanced further by PTH₁₋₃₄ treatment. We attributed these effects to delayed callus remodeling. It, therefore, seems likely that delayed remodeling may also be responsible for the increase in fracture biomechanical properties observed at day 42 in the *Cox-2*^{-/-} mice.

This study was performed in a stabilized tibia osteotomy model that is highly reproducible but has a relatively lower level of callus formation compared to an unstabilized femoral fracture model. Overall, this model shows that PTH modulates several events during bone repair, including the regulation of progenitor cell proliferation, callus formation, and the rate and mechanical properties of the fracture repair process. The effect of PTH is observed in both wild type mice as well as in COX-2 mutant mice. Therefore, this study will help our understanding of the poor healing process under reduced COX-2 conditions, such as aging, and PTH₁₋₃₄ treatment may eventually be a therapeutic option to enhance fracture healing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Fracture healing, as assessed using a murine stabilized tibia fracture model, was impaired in *Cyclooxygenase-2* (*Cox-2*)-deficient mice.
2. Teriparatide (human PTH₁₋₃₄) improved callus volume and biomechanical properties during the early phases of healing in *Cox-2*-deficient tibial fractures.
3. PTH₁₋₃₄ increased periosteal progenitor cell proliferation, bone callus formation, and callus remodeling.

Figure 1. PTH₁₋₃₄ enhances fracture callus formation in *Cox-2* deficient mice

(A) Continuous radiographs of representative wild type (WT), *Cox-2*^{+/-} and *Cox-2*^{-/-} mice treated with normal saline (control) or PTH₁₋₃₄ at 10 and 14 days post-fracture. At day 14, the fracture line was no longer visible in WT and *Cox-2*^{+/-} control-treated mice, but remained visible in *Cox-2*^{-/-} control mice. PTH₁₋₃₄ enhanced fracture callus formation in all genotypes on days 10 and 14. (B) Fracture specimens were harvested, and high-resolution μ CT scans were performed on WT, *Cox-2*^{+/-} and *Cox-2*^{-/-} mice with normal saline or PTH₁₋₃₄ (n=3–9). Representative scans are shown for days 7, 10, 14, and 21 for calcified external callus. Mean calculated external bony callus volume was obtained (C). Between day 10 and 21, PTH₁₋₃₄ significantly increased the external bony callus volumes in all genotypes. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using heteroscedastic t-tests. *P<0.05 and **P<0.01 v.s. wild type control or #P<0.05 and ##P<0.01 v.s. untreated genotype-matched control.

Figure 2. PTH₁₋₃₄ increased biomechanical properties in *Cox-2* deficient mice

Fractured tibias were retrieved upon animal sacrifice at 10, 14, 21, and 42 days and tested in torsion at 1°/sec to determine the ultimate torque (A), yield torque (B), torsional rigidity (C), and toughness (D). Intermittent PTH₁₋₃₄ treatment improved the biomechanical torsion properties of fractured tibias in all genotypes. Data are presented as mean ± SEM. Statistical analysis was performed using heteroscedastic t-tests. **P*<0.05 and ***P*<0.01 v.s. wild type (WT) control or #*P*<0.05 and ##*P*<0.01 v.s. untreated genotype-matched control.

Figure 3. PTH₁₋₃₄ rescues callus formation in *Cox-2* deficient mice

Representative Alcian blue hematoxylin/Orange G eosin sections of wild type (WT) and *Cox-2*^{-/-} fractures with normal saline or PTH₁₋₃₄ treatment at various time points after fracture are shown (A, Scale bar: 1mm). Histology showed that PTH₁₋₃₄ rescued an impaired cartilaginous callus formation at 7 days after fracture in *Cox-2*^{-/-} mice. In contrast, WT mice showed a delayed completion of endochondral bone formation by PTH₁₋₃₄ treatment. Histomorphometric analysis of total callus, mesenchyme, bone, and cartilage areas was completed in WT, *Cox-2*^{+/+} and *Cox-2*^{-/-} mice (n=4), with 3 levels analyzed per

specimen. Histomorphometry showed an increase of all parameters by PTH₁₋₃₄ treatment (B-E). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using heteroscedastic t-tests. *P<0.05 and **P<0.01 v.s. wild type (WT) control or #P<0.05 and ##P<0.01 v.s. untreated genotype-matched control.

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Figure 4.

Real-time PCR analyses were performed using RNA collected from wild type (WT), *Cox-2*^{+/-} and *Cox-2*^{-/-} fracture callus with normal saline or PTH treatment on day 3, 5, 7, 10, 14 and 21 post-fracture. *Sox9*, *Col2a1*, and *Col10a1* (chondrogenesis related genes) and *Runx2*, *Osterix*, and *Bglap* (osteogenesis related genes) are normalized to β -actin expression and shown respectively. Each time point included at least 4 fractured samples. Gene expression showed an increase in hypertrophic cartilage marker gene (*Col10a1*) and mature osteoblasts marker gene (*Bglap*) in PTH₁₋₃₄ treated *Cox-2*^{-/-} fractures. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using heteroscedastic t-tests. *P<0.05 and **P<0.01 v.s. wild type (WT) control or #P<0.05 and ##P<0.01 v.s. untreated genotype-matched control.

Figure 5. PTH₁₋₃₄ improves impaired periosteal cell proliferation during the early stage of fracture healing

Representative PCNA staining sections of wild type (WT), *Cox-2*^{+/-} and *Cox-2*^{-/-} fractures with normal saline or PTH₁₋₃₄ treatment at days 5 and 7 after fracture are shown (A, * the original cortex bone, Scale bar: 250μm) The percentage of PCNA positive cells and total cell number on the periosteal area were noted at 3, 5, and 7 days after fracture (B). *Ccnd1* expression was measured by realtime RT-PCR (C). Data are presented as mean ± SEM. Statistical analysis was performed using heteroscedastic t-tests. *P<0.05 and **P<0.01 v.s. wild type (WT) control or #P<0.05 and ##P<0.01 v.s. untreated genotype-matched control.

Figure 6. The effect of PTH 1_{-34} on osteoclast formation during fracture healing

Representative TRAP staining sections of wild type (WT), *Cox-2*^{+/-} and *Cox-2*^{-/-} fractures with normal saline or PTH₁₋₃₄ treatment at day 14 after fracture are shown (A, * the original cortex bone, Scale bar: 250μm). TRAP positive cells per total callus area are shown (B).

Time course change of *Tracp* gene expression is measured by realtime RT-PCR (C). Data are presented as mean ± SEM. Statistical analysis was performed using heteroscedastic t-tests.

*P<0.05 and **P<0.01 v.s. wild type (WT) control or #P<0.05 and ##P<0.01 v.s. untreated genotype-matched control.