

Delayed Short-Course Treatment With Teriparatide (PTH_{1–34}) Improves Femoral Allograft Healing by Enhancing Intramembranous Bone Formation at the Graft–Host Junction

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ABSTRACT

Clinical management of critical bone defects remains a major challenge. Despite preclinical work demonstrating teriparatide (PTH_{1–34}) effectiveness in small animals, inconclusive data from clinical trials have raised questions of dose and regimen. To address this, we completed a comprehensive study in the murine femoral allograft model, to assess the effects of dose (0.4, 4, and 40 µg/kg/day) and various treatment regimens on radiographic, histologic, and biomechanical healing at 2, 4, and 9 weeks. Only the high dose (40 µg/kg) of PTH_{1–34} demonstrated significant effects when given daily over 9 weeks. Remarkably, equivalent biomechanical results were obtained with delayed, short treatment from 2 to 6 weeks that did not induce a significant increase in endochondral bone formation and callus volume. In contrast, PTH_{1–34} treatment from 1 to 5 weeks postop demonstrated similar osteogenic effects as immediate daily treatment for 9 weeks, but failed to achieve a significant increase in biomechanics at 9 weeks. MicroCT and histologic analyses demonstrated that the 2-week delay in treatment allowed for timely completion of the endochondral phase, such that the prominent effects of PTH_{1–34} were enhanced intramembranous bone formation and remodeling at the graft–host junction. These findings support the potential use of PTH_{1–34} as an adjuvant therapy for massive allograft healing, and suggest that there may be an ideal treatment window in which a short course is administered after the endochondral phase to promote osteoblastic bone formation and remodeling to achieve superior union with modest callus formation. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: BONE; ALLOGRAFT; PARATHYROID HORMONE (PTH); BIOMECHANICS; MICROCOMPUTED TOMOGRAPHY (MICROCT)

Introduction

Although most orthopedic fractures heal, the clinical management of critical (>3 cm) segmental defects continues to face major challenges for both amputation and limb salvage approaches, which have high rates of self-reported disability (40%–50%) and continue to worsen over time.^(1,2) Despite lacking osteogenic and remodeling capacity, devitalized cortical allografts remain a major option for reconstructive surgery because no equivalent alternatives exist. However, the limited new bone formation and lack of remodeling associated with massive allograft healing are directly associated with the 23% to 43% clinical failure rate caused by nonunion (27%–34%), late graft fracture (24%–27%), and infection (9%–16%).⁽³⁾ Thus, the quest for a practical adjuvant therapy for massive allograft surgery remains a high priority.

Intermittent administration of PTH_{1–34}, an active recombinant human peptide sequence of parathyroid hormone, has been proven to increase skeletal bone mass in osteoporotic patients.⁽⁴⁾ Based on these anabolic properties, several groups have demonstrated that PTH_{1–34} is effective in small animal models of fracture healing.^(5–10) Moreover, clinical case reports of off label use of PTH_{1–34} to heal delayed and nonunion fractures have suggested that the drug could be effective as an adjuvant for challenging bone healing situations.^(11,12) Unfortunately, a recent phase 2 clinical trial of distal radial fractures in postmenopausal women failed to meet its primary prospective endpoint with 40 µg/day teriparatide, although the study did show that 20 µg/day teriparatide accelerated the time to radiographic healing from 9.1 to 7.4 weeks versus saline controls ($p = 0.006$).⁽¹³⁾ The efficacy of the therapy in this study, however, was confounded by a fracture model that would normally

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heal in the placebo group irrespective of treatment, which raises several questions that warrant further investigation of PTH₁₋₃₄ dose and regimen in challenging preclinical models of bone healing.

One preclinical model of challenging bone healing that has emerged as a useful tool to understand the biological issues associated with delayed healing and nonunions is the murine femoral allograft model.⁽¹⁴⁾ Similar to the clinical situation, femoral allografts in mice heal only via creeping fracture callus from the host that is mediated by endochondral ossification, and there is very little remodeling of the necrotic graft because of the absence of osteoclasts on the cortical surface of the allograft. Furthermore, murine allografted femurs achieve <50% of the torsional biomechanical properties of normal, ungrafted controls,⁽¹⁵⁾ which is the primary outcome measure of reconstructive surgery for segmental defects in preclinical models. Live bone graft studies have demonstrated that the lack of intramembranous bone formation is because of the absence of periosteal cells, which are responsible for the angiogenic, osteogenic, and remodeling responses during allograft healing.^(16,17) Although these signals can be supplied exogenously via recombinant virus mediated gene transfer or cell therapy,^(16,18,19) PTH₁₋₃₄ adjuvant therapy offers the potential for a more practical solution for this problem.

To better understand the potential of PTH₁₋₃₄ as an adjuvant therapy for structural allograft healing, several questions about optimal dosing regimen must be addressed. With regard to dose, there is a significant discrepancy (approximately 100-fold) between the doses used in the rodent models (typically 40 $\mu\text{g}/\text{kg}/\text{day}$),⁽⁵⁻¹⁰⁾ versus the approved clinical dose of 20 μg per day total (which in a 50-kg to 80-kg person translates to 0.25 $\mu\text{g}/\text{kg}/\text{day}$ to 0.4 $\mu\text{g}/\text{kg}/\text{day}$). This discrepancy suggests that either the higher metabolic activity in small mammals requires higher dosing (on a per weight basis), and/or that efficacious dosing for bone healing is greater than that of anabolic therapy for generalized osteopenia. Moreover, questions about the ideal duration and timing of PTH₁₋₃₄ therapy regimen for bone healing after reconstructive surgery have not been addressed. Although osteoporosis therapy is indicated up to 24 months, it is unlikely that PTH₁₋₃₄ treatment beyond a few months could significantly influence the dynamics of fracture healing. Given the potential safety concerns associated with long-term use of the drug, in addition to its high cost and the inconvenience of daily subcutaneous injections, identifying the minimum treatment duration required for efficacy is critical for this indication of PTH₁₋₃₄ therapy. Finally, there is a question as to the ideal timing of PTH₁₋₃₄ therapy after surgery. Because PTH has multiple mechanisms of action during bone repair via direct and indirect effects on multiple cell types during the initial inflammatory, intermediate osteogenic, and late remodeling stages of bone healing, it is important to elucidate the effective window of therapy that achieves the maximum benefit on allograft healing. Therefore, a systematic study to investigate the appropriate clinically translatable dosing regimen of PTH₁₋₃₄ needs to be conducted. To this end, we examined the effects of various doses, timing, and duration of PTH₁₋₃₄ therapy on radiographic, histologic, and biomechanical properties of murine femoral allograft healing.

Methods and Materials

Preparation of decellularized allografts

Devascularized femoral allografts were prepared from femurs of 9-week-old female ICR mice as previously described.⁽¹⁴⁾ The donor femurs were cut to 4-mm middiaphyseal allografts, bone marrow was flushed using saline from a syringe, then the allografts were bathed in 70% ethanol for 3 hours and washed in phosphate-buffered saline (PBS) before storing at -70°C for at least 1 week before they were used.

Femoral allograft reconstruction surgery

All animal studies were performed in accordance with protocols approved by the University of Rochester's Committee on Animal Resources. Femoral allograft surgeries were performed as previously described.⁽¹⁴⁾ Briefly, devascularized ICR allografts were implanted into a 4-mm defect created in the middiaphysis of the left femur of a recipient 9-week-old female C57Bl/6 mouse using a 10-mm diameter, 0.15-mm thick diamond-sintered rotary saw (Brasseler USA, Inc., Savannah, GA, USA), and secured in place with an intramedullary stainless steel pin (Fig. 1A). Daily subcutaneous injections of teriparatide (human PTH₁₋₃₄) (ForteoTM, Eli Lilly and Co., Indianapolis, IN, USA) or saline (control) was performed according to the dosing regimens described in Figure 1. Group names were defined by dose, timing of initial treatment, and duration as follows. Three daily doses of PTH₁₋₃₄ were investigated (L = low dose of 0.4 $\mu\text{g}/\text{kg}/\text{day}$; M = middle dose of 4 $\mu\text{g}/\text{kg}/\text{day}$; and H = high dose of 40 $\mu\text{g}/\text{kg}/\text{day}$). The treatment regimen was defined by the timing of the initial dose (0 or 1 day after the surgery; and 1, 2, or 4 weeks postop), and the timing of treatment withdrawal (2, 3, 4, 5, 6, 8, or 9 weeks postop).

Experiment 1: dose response study

Daily doses of PTH₁₋₃₄ (0.4, 4, and 40 $\mu\text{g}/\text{kg}/\text{day}$) or saline were administered to the mice ($n = 15$ per group) via subcutaneous injections from 1 day after the surgery for 4 weeks (Fig. 1B). The mice were then maintained without therapy for another 5 weeks before sacrificed at 9 weeks postsurgery. Healing of grafted femurs was monitored by weekly X-rays (Supplemental Fig. 1), and the grafted femurs were harvested and subjected to microcomputed tomography (microCT) analysis. Then, 10 femurs were used for torsion testing, and the remaining 5 femurs were used for histology.

Experiment 2: duration and timing study

Administration of high-dose (40 $\mu\text{g}/\text{kg}/\text{day}$) PTH₁₋₃₄ was commenced either 1 day after the surgery (immediately), or at 1, 2, or 4 weeks postsurgery. The dosing periods were either 2 or 4 or 9 weeks (Fig. 1C). The saline-treated group as a control and the H-0-4 group in experiment 1 were included in this analysis. A total of 15 mice in each group were sacrificed at 9 weeks postsurgery and subjected to microCT ($n = 15$), biomechanical testing ($n = 10$), and histology ($n = 5$) as depicted in Figure 1C.

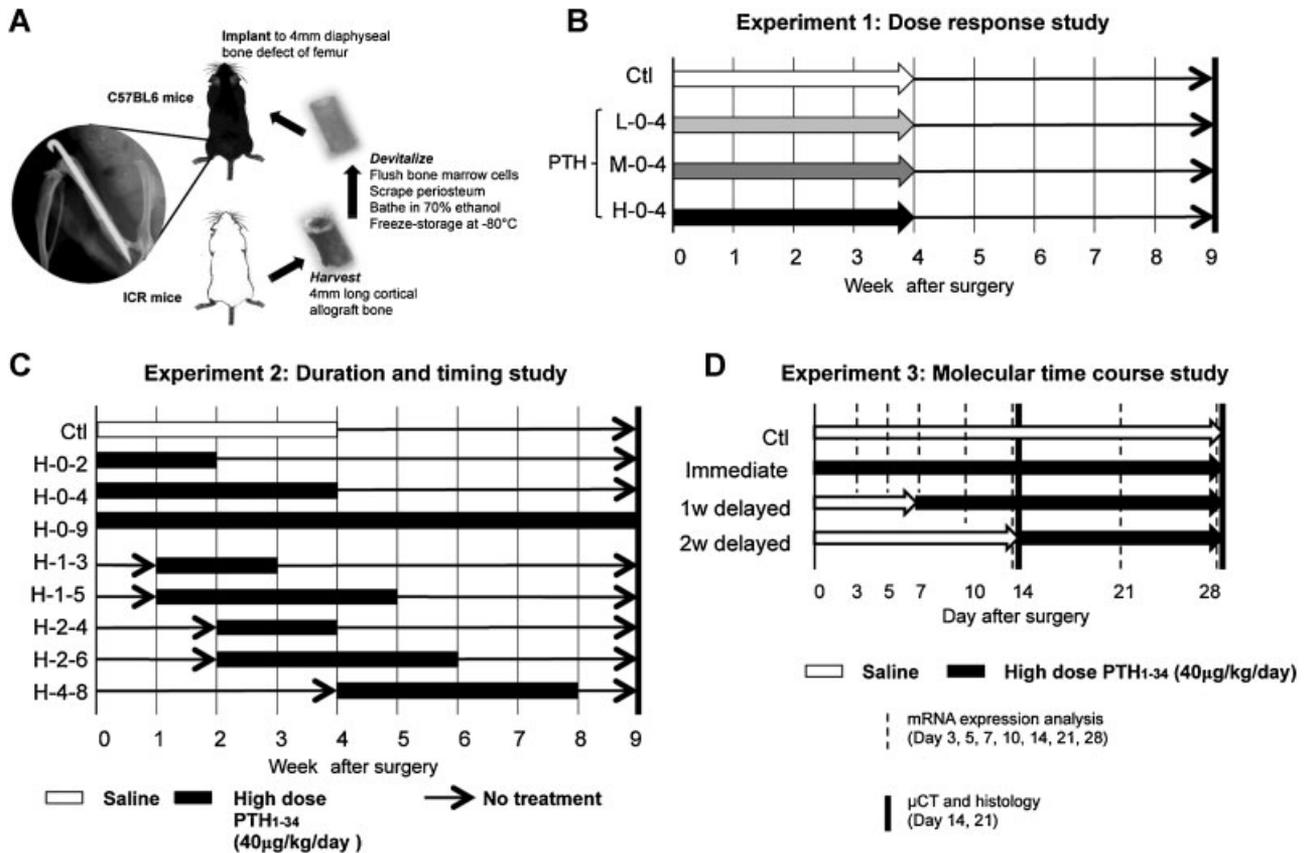


Fig. 1. Experimental model and design. (A) Schematic diagram of murine femur allograft surgery. Allografts were harvested from ICR mice and devitalized. Processed allograft were then implanted to 4-mm diaphyseal defects of left femurs of C57bl/6 mice and secured with intramedullary pins. Panels B–D show the experimental groups and treatment schedules for the dose–response study, duration and timing study, and molecular time course study, respectively. Group name indicates: dose of daily PTH_{1–34} injection (L: low, M: middle, and H: high) timing of commencing treatment (week) timing withdrawal of treatment (week). Mice were sacrificed at week 9 in experiments 1 and 2 and at various time points up to 28 days in experiment 3.

Experiment 3: PTH effects on gene expression during allograft healing

To elucidate the early mechanistic differences between immediate and delayed PTH_{1–34} therapy on allograft healing we evaluated the healing response at 2 or 4 weeks postsurgery. Three PTH_{1–34} dosing regimens starting either 1 day after the surgery (immediately), 1 week after surgery (1w delayed), or 2 weeks after surgery (2w delayed) were compared with the saline treatment control group as depicted in Figure 1D. A total of 10 mice in each group were assessed by microCT and histology ($n = 5$ per group per time point). In addition, three mice per group were sacrificed at various times (0, 3, 7, 10, 14, 21, and 28 days) postsurgery for mRNA expression analysis.

MicroCT imaging of bone formation and union

The femurs were harvested from euthanized mice, disarticulated at the hip and knee joints, and the intramedullary pins were carefully removed. The specimens for biomechanical testing were stored at -20°C , and the specimens for histology were fixed in 10% neutral buffered formalin and stored in 70% ethanol, before microCT imaging. The specimens were scanned at 12.5 microns isotropic resolution using the VivaCT 40 (Scanco Medical AG, Bassersdorf, Switzerland) as previously described.⁽¹¹⁾

From these 3D images, the callus bone volume was measured by manual segmentation, followed by standardized thresholding at a grayscale corresponding to $750\text{ mgHA}/\text{cm}^3$ based on a phantom of known HA concentrations. The Union Ratio, a measure of allograft osseointegration, which is based on the minimum graft surface area fraction upon which mineralized callus had formed, was also calculated as recently described.⁽¹¹⁾

Biomechanical testing and failure mode analysis

Immediately after microCT imaging, the torsional biomechanical properties of the grafted femurs were determined as previously described,⁽²⁰⁾ using an EnduraTec TestBench™ system (200 N.mm torque cell; Bose Corporation, Minnetonka, MN, USA) at a rate of $1^{\circ}/\text{s}$. Yield torque (T_{Yield}), ultimate torque (T_{Ult}), torsional rigidity (TR) and work to failure were determined for each specimen. After torsion testing, the specimens were X-rayed to analyze the mode of failure as previously described.⁽²⁰⁾

Histology and histomorphometric analyses

After microCT, femurs were decalcified in EDTA. At least two nonconsecutive $3\text{-}\mu\text{m}$ paraffin embedded midsagittal sections were stained with either alcian blue/hematoxylin/orange G (AB/OG) or tartrate resistant acid phosphatase (TRAP). Cartilage area

and osteoclast number (Oc.N) were measured in week 2 and week 4 samples in experiment 3 as described previously.⁽¹⁴⁾ Briefly, cartilage area was measured by alcian blue stained area. Oc.N was determined from sections stained for TRAP by counting the number of TRAP positive cells. Oc.N inside the callus area (Oc.N_{callus}) and on the surface of allograft (Oc.N_{graft}) was evaluated separately. The mean values from the two nonconsecutive midsagittal sections represented the value for one mouse.

Gene expression analysis

The mRNA expression levels of various genes within the tissue at graft–host junction were measured using real-time, reverse transcription-polymerase chain reaction (RT-PCR). The 3-mm-long callus tissue that formed at graft–host junctions was harvested at postoperative days 3, 5, 7, 10, 14, 21, and 28 as shown in Figure 1D. Tissues were flushed with DEPC-treated PBS to wash out bone marrow cells. The samples were frozen in liquid nitrogen and homogenized using a dismembrator. Total RNA was extracted from the samples using the TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA). For cDNA synthesis, 1 µg RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was performed using a 20-µL reaction volume. Signals were detected using the PerfeCTa SYBR Green FastMix (Quanta BioSciences, Inc., Gaithersburg, MD, USA) with gene-specific primers. Gene-specific primers of type II collagen (*colla2*), type X collagen (*colXa1*), vascular endothelial growth factor-*a* (*vegfa*), matrix metalloproteinase 9 (*mmp9*), type I collagen (*colla1*), osteocalcin (*oc*), cathepsin K (*ctsk*), tartarate resistant acid phosphatase (*trap*), and β -actin as a housekeeping gene are shown in supplemental Table 1. The relative mRNA expression of each targeted gene was normalized by the cycle threshold values of β -actin.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's or Dunnett's post hoc multiple comparison tests were performed to assess differences among PTH_{1–34} treatment groups and compared with the control groups, respectively. Values of $p \leq 0.05$ were used to detect significant differences.

Results

Dose effects of PTH_{1–34} on murine femoral allograft healing

To establish the effective dose of the therapy for murine allograft reconstruction we injected animals with 0 (saline control), 0.4, 4, or 40 µg/kg/day of PTH_{1–34} for 4 weeks after surgery. As restoration of biomechanical integrity of the injured long bone is the primary goal of structural allografting, we assessed this outcome 9 weeks after surgery to allow for adequate healing for torsion testing.⁽²⁰⁾ High-dose PTH_{1–34} treatment (H-0-4) increased the grafted femur yield torque (T_{Yield}), ultimate torque (T_{Ult}), and torsional rigidity (TR) to 2.6, 2.2, and 1.9 times those in the control group (Table 1). In contrast, middle- (M-0-4) and low-dose (L-0-4) PTH_{1–34} showed equivalent values to those in the control group. Assessment of the mode of failure confirmed these results, as control and low dose groups resulted in preunion at a rate of 40% and 50%, respectively, but most importantly never achieved mature union by the end of the study. In contrast, none of the allografts in the high-dose group resulted in preunion, whereas 10% of those achieved mature union (Supplemental Table 2)

Effects of treatment duration of PTH_{1–34} on biomechanical parameters of femoral allograft healing

To assess the differential effects of duration of PTH_{1–34} therapy on allograft healing in our model, we evaluated high-dose

Table 1. Biomechanical (Torsional) Properties of the Grafted Femurs at 9 Weeks, Presented as Mean ± Standard Deviation

	Yield Torque (TYield)	Ultimate Torque (TUlt)	Torsional Rigidity	Work to TUlt
	N.mm	N.mm	N.mm/rad.mm ⁻¹	N.mm.Rad/mm
Saline Control	2.9 ± 2.7	5.3 ± 5.0	411.4 ± 395.8	0.08 ± 0.08
Dose and duration effects for immediate PTH _{1–34} treatment				
L-0-4	3.0 ± 4.1 ($p = 1.00$)	4.1 ± 5.8 ($p = 0.99$)	312.9 ± 334.1 ($p = 0.99$)	0.06 ± 0.09 ($p = 0.99$)
M-0-4	2.7 ± 2.0 ($p = 1.00$)	6.1 ± 4.7 ($p = 1.00$)	383.5 ± 283.7 ($p = 1.00$)	0.10 ± 0.07 ($p = 1.00$)
H-0-2	8.7 ± 2.7 ($p = 0.07$)	12.6 ± 4.1 ^a ($p < 0.05$)	672.1 ± 228.0 ($p = 0.51$)	0.22 ± 0.09 ($p = 0.10$)
H-0-4	7.5 ± 3.7 ($p = 0.23$)	11.7 ± 4.4 ($p = 0.11$)	777.1 ± 262.1 ($p = 0.15$)	0.19 ± 0.18 ($p = 0.28$)
H-0-9	10.5 ± 7.4 ^a ($p < 0.01$)	13.3 ± 7.1 ^a ($p < 0.05$)	707.2 ± 350.7 ($p = 0.36$)	0.24 ± 0.13 ^a ($p < 0.05$)
High dose, 1-week delayed PTH _{1–34} treatment				
H-1-3	6.7 ± 4.8 ($p = 0.46$)	8.3 ± 4.9 ($p = 0.87$)	583.2 ± 227.9 ($p = 0.91$)	0.10 ± 0.05 ($p = 1.00$)
H-1-5	8.1 ± 5.0 ($p = 0.13$)	10.3 ± 5.6 ($p = 0.31$)	695.5 ± 401.5 ($p = 0.41$)	0.19 ± 0.08 ($p = 0.25$)
High dose, 2-week delayed PTH _{1–34} treatment				
H-2-4	8.1 ± 7.1 ($p = 0.06$)	12.5 ± 8.5 ^a ($p = 0.05$)	644.6 ± 466.7 ($p = 0.64$)	0.22 ± 0.13 ($p = 0.07$)
H-2-6	9.9 ± 6.6 ^a ($p < 0.05$)	14.0 ± 7.7 ^a ($p < 0.01$)	840.7 ± 423.7 ($p = 0.07$)	0.26 ± 0.15 ^a ($p < 0.01$)
High dose, 4-week delayed PTH _{1–34} treatment				
H-4-8	2.8 ± 3.1 ($p = 1.00$)	4.7 ± 4.3 ($p = 1.00$)	228.3 ± 292.7 ($p = 0.89$)	0.15 ± 0.19 ($p = 0.79$)

Superscript a indicates significant differences from the saline control when $p < 0.05$ (p -values shown in parentheses).

treatment regimens commencing immediately out to 2, 4, or 9 weeks postsurgery, and evaluated the biomechanical properties at 9 weeks. The H-0-9 group served as the positive control for this study, as all tested parameters of healing were significantly ($p < 0.05$) greater than saline-treated controls. Contrary to our expectation, we found that immediate treatment for only 2 weeks (H-0-2) was sufficient to significantly increase the grafted femur T_{Ult} to 2.4 times that in the control group ($p < 0.05$), and was not significantly different than the H-0-9 group (Table 1). We also observed a trend in which H-0-2 group demonstrated a threefold increase in T_{Yield} and 1.6-fold increase in TR of grafted femur over saline control, although these differences were not statistically significant. The 4-week (H-0-4) and 9-week (H-0-9) treatment groups were equivalent in biomechanical properties to 2 weeks-long (H-0-2) treatment, indicating that longer treatments do not further improve allograft healing in terms of bone strength. The conclusion was also supported by the failure mode analysis, which demonstrated that the additional 5 weeks of PTH₁₋₃₄ treatment in the H-0-9 group resulted in only a 20% increase in the incidence of mature union compared with the H-0-4 group (Supplemental Table 2).

Effects of delayed PTH₁₋₃₄ treatment on biomechanical parameters of femoral allograft healing

To assess the efficacy of delayed PTH₁₋₃₄ treatment on allograft healing in our model, we evaluated the effects of commencing the treatment after 1, 2, or 4 weeks postreconstruction for 2 or 4 weeks thereafter. We found the treatment 4 weeks after surgery (H-4-8) to be completely ineffective, as no differences were observed compared with saline-treated controls. Interest-

ingly, 1 or 2 week delayed treatments resulted in equivalent biomechanical properties to the positive control H-0-9, regardless of the duration of treatment. However, the most remarkable results from this study came from the H-2-6 treatment group, which demonstrated significant increases in all parameters tested compared with saline controls, and achieved equivalent biomechanical outcomes to the H-0-9 treatment group.

Although 9 weeks postsurgery is ideal for assessing drug effects on torsional biomechanics in this model,⁽¹¹⁾ this time point proved to be too challenging for quantitative radiology and histomorphometry, because of the extensive remodeling that prohibits accurate segmentation of the graft from the host bone, and remodeling of callus and new periosteal bone. Additionally, unsegmented microCT analyses of the grafted femurs demonstrated that no differences in bone volume or bone mineral density at 9 weeks could be observed among the groups (data not shown). However, the 3D microCT rendering of the grafted femurs demonstrated gaps and irregular cortical surfaces at the graft–host junction of the control, L-0-4, and M-0-4 groups (Fig. 2) that were not observed in the H-0-4 group, demonstrating that only the high dose has effects on bone healing in this model.

Effects of PTH₁₋₃₄ treatments on radiographic and histologic parameters of femoral allograft healing

To better understand the mechanism responsible for these drug effects on biomechanical properties, we analyzed microCT images and histology at 2 and 4 weeks postsurgery (Fig. 1D). These analyses were limited to only the different high-dose treatment regimens, because the low- and middle-dose

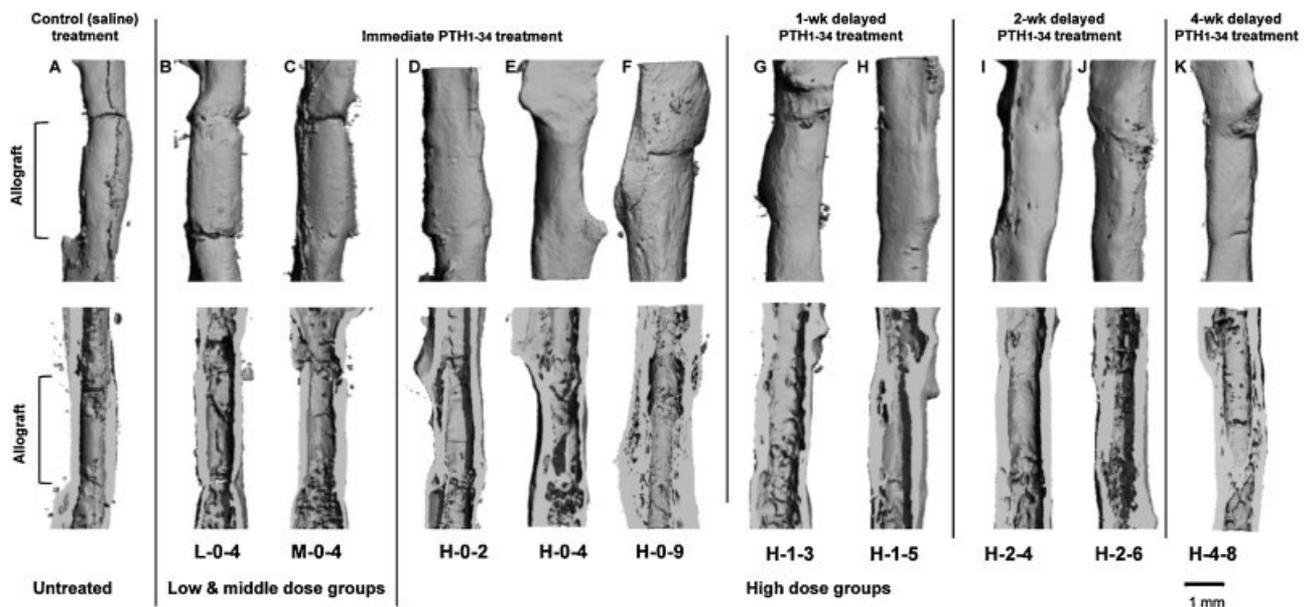


Fig. 2. Effects of dose and regimen of PTH₁₋₃₄ treatment on graft–host osseointegration at 9 weeks postsurgery. MicroCT rendering of grafted femurs (full views in *top panel* and coronal sections in *bottom panel*) at 9 weeks postsurgery in experiments 1 and 2 demonstrated that allograft healing and incorporation is enhanced by various regimens of high dose (40 $\mu\text{g}/\text{kg}/\text{day}$) PTH₁₋₃₄ therapy (D–J), whereas low and middle doses (0.4 and 4 $\mu\text{g}/\text{kg}/\text{day}$) still showed discontinuities at the graft–host junctions (B and C) similar to untreated controls (A). Delayed PTH₁₋₃₄ treatment up to 1 or 2 weeks postsurgery still improved allograft integration with the host, regardless of the duration of treatment (G–J), and were grossly comparable to immediate treatment. Only the 4-week delayed PTH₁₋₃₄ treatment (K) did not show significant effects on graft–host union compared with untreated controls.

treatments were not biomechanically efficacious. Interestingly, although immediate PTH₁₋₃₄ treatment significantly increased callus volume at 2 weeks (Fig. 3C, H) compared with controls (Fig. 3A, H), this increase was not observed in the 1 week delayed treatment group (Fig. 3E, H), and was not significant at 4 weeks because of remodeling. No treatment related effects on the callus BMD were detected at either 2 or 4 weeks postsurgery (Fig. 3I). However, both the immediate and 1-week delayed treatment groups displayed significant increases in their Union Ratios by 4 weeks (Fig. 3J), which demonstrates that this parameter of osseointegration is not related to callus volume, as we have previously reported.^(11,15) The results of this experiment also demonstrated that whereas a 2-week delay in the PTH₁₋₃₄ treatment did not increase the Union Ratio per se compared with saline controls (Fig. 3J), microCT data at 9 weeks showed remarkable end-to-end integration in the treated specimens, regardless of the delay, which underscores that the long-term healing is independent of callus size.

Histological analyses of the healing allografts harvested 2 and 4 weeks postsurgery also provided evidence for PTH₁₋₃₄-induced osseointegration independent of callus size (Fig. 4). At 2 weeks, there was a small, albeit statistically insignificant, increase in the cartilage area with the immediate PTH₁₋₃₄ treatment compared with the saline controls and 1 week delayed treatment (Fig. 4A-C, Table 2). Moreover, there was no significant evidence of persistent cartilage at 4 weeks (Fig. 4D-G, Table 2), regardless of the treatment. Furthermore, there were mild increases in the osteoclast number density at the surface of the treated allografts at 2 weeks, which increased by 4 weeks in all PTH₁₋₃₄ treatment groups (Fig. 4, Table 2). Furthermore, the osteoclast number density in the callus was significantly higher than that on the allograft surface at 2 weeks, regardless of the treatment. At 4 weeks, the osteoclast number density remained increased in the treated groups, but decreased significantly in the saline treated controls (Table 2).

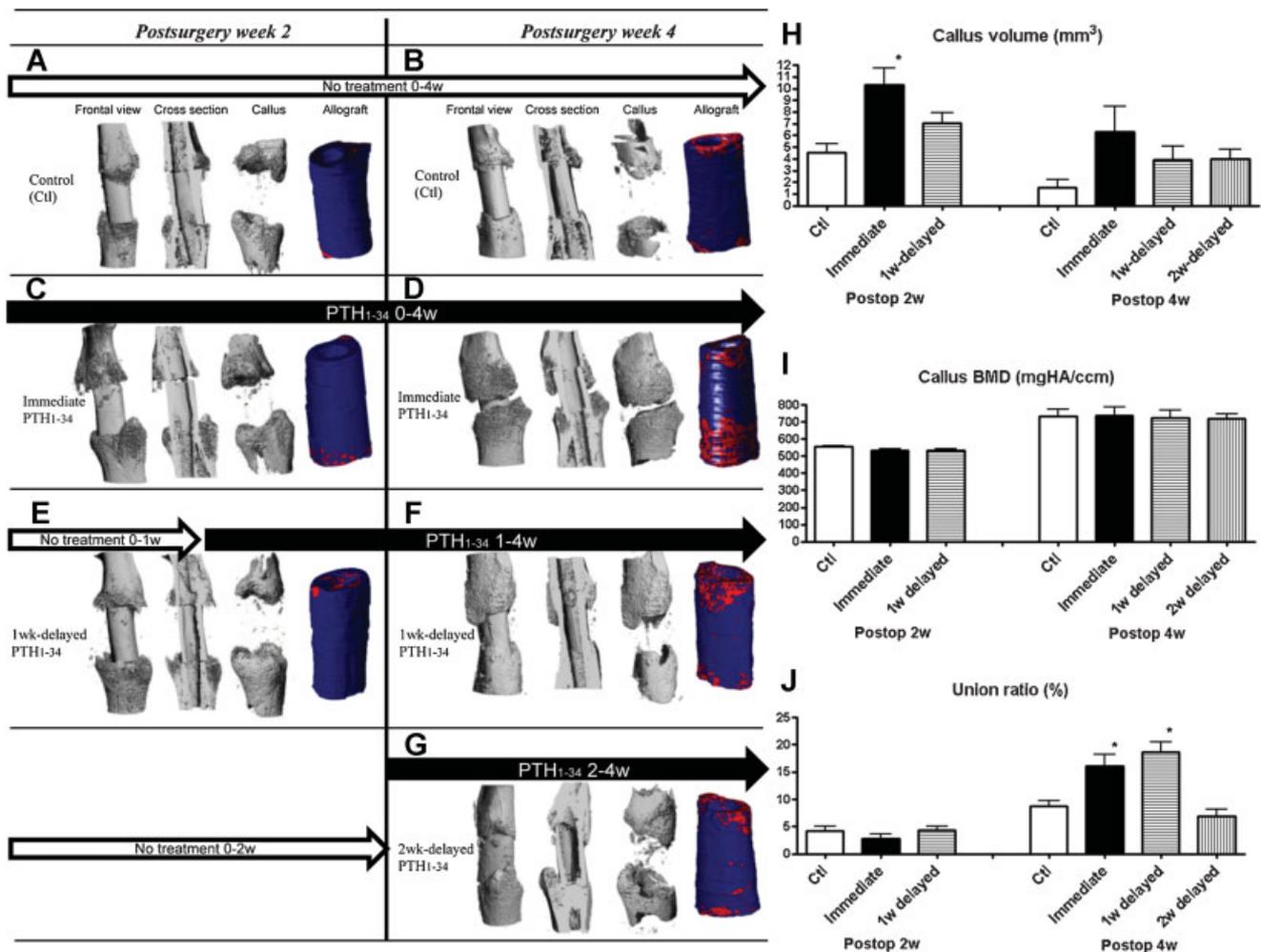


Fig. 3. Effects of treatment regimen of high-dose PTH₁₋₃₄ on graft-host osseointegration at 2 and 4 weeks postsurgery. Representative microCT rendering of femoral allografts and surrounding callus at 2 weeks (A, C, and E) and 4 weeks (B, D, F, and G) depicting 3D frontal view, coronal cross-sectional view, segmented callus, and the segmented allograft, respectively, of grafts treated with saline (controls) or 40 μg/kg/day PTH₁₋₃₄ commencing immediately or after a 1- or 2-week delay. The callus volume (H) and bone mineral density (I) are quantified and plotted for the various treatment regimens. Allograft images are artificially colored in blue and red to calculate the union ratio (J), where blue represents bare allograft surface not in contact with surrounding mineralized callus, while red areas indicate regions of graft union with mineralized callus from the host. Bar plots (H-J) represent means and SEM. Asterisks indicate significant differences versus controls ($p < 0.05$).

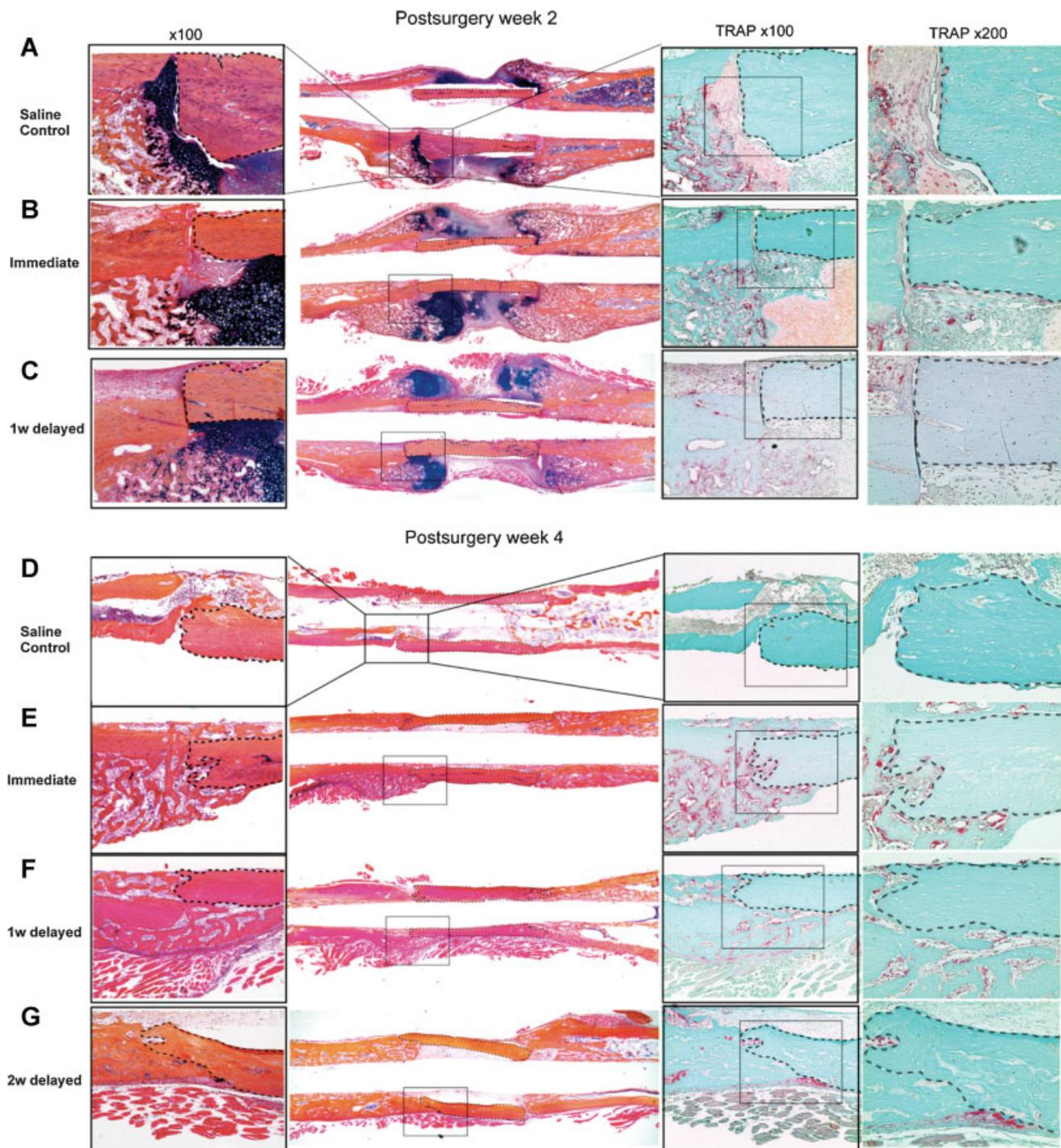


Fig. 4. Effects of treatment regimen of high-dose PTH₁₋₃₄ on cartilage and bone formation, and osteoclast activity at 2 and 4 weeks postsurgery. Micrographs showing representative sagittal sections of grafted femurs treated with saline (A and D) and 40 $\mu\text{g}/\text{kg}$ PTH₁₋₃₄ commencing immediately postsurgery (B and E), after a 1-week delay (C and F), or a 2-week delay (G), respectively. *Insets on the left-hand side* show higher magnification ($\times 100$) of the graft–host junction (boxed area) stained with alcian blue/hematoxylin/orange G, while *insets on the right-hand side* show the same view stained with TRAP at $\times 100$ and $\times 200$, respectively. Note that cartilage area, which reflects chondrogenesis during endochondral bone formation stage, was increased by immediate PTH₁₋₃₄ treatment (B) compared with untreated control (A) and the 1-week delayed treatment (C). Note also that compared with controls (D) graft–host integration was improved at 4 weeks by PTH₁₋₃₄ treatment regardless of the timing of the treatment (E–G). Although a number of osteoclasts were observed in the callus area at 2 weeks in all the groups including control, the number of osteoclasts in callus area decreased from 2 to 4 weeks with the progression of callus remodeling. At 4 weeks, only a few osteoclasts could be found in the callus area in the control specimens, whereas a much greater number of osteoclasts could still be observed in callus area in the PTH₁₋₃₄ treatment groups. Osteoclasts were rarely found on the surface of devitalized allograft (not shown), but some could be observed at the region of allograft where callus enveloped.

Table 2. Histomorphometry Analysis of Allograft Healing

Parameter	Postsurgery Week 2			Postsurgery Week 4			
	Saline Control	PTH ₁₋₃₄ -Immediate	PTH ₁₋₃₄ -1w Delayed	Saline Control	PTH ₁₋₃₄ -Immediate	PTH ₁₋₃₄ -1w Delayed	PTH ₁₋₃₄ -2w Delayed
Cartilage area (mm ²)	0.40 ± 0.23	1.09 ± 0.85	0.63 ± 0.39	0.02 ± 0.03	0.09 ± 0.18	0.05 ± 0.04	0.04 ± 0.03
Osteoclast number on graft surface (/section)	2.3 ± 1.9	8.6 ± 3.7 ^a	5.3 ± 4.3	5.0 ± 4.5	16.4 ± 7.6	20.6 ± 8.7 ^a	17.8 ± 8.3
Osteoclast number in callus area (/mm ²)	55.0 ± 12.9	59.4 ± 14.7	64.7 ± 17.0	3.7 ± 3.9	33.2 ± 14.6	33.6 ± 22.4	38.78 ± 17.2 ^a

Values are mean ± SD ($n = 5$ for each group at week 2 and $n = 4$ for each group at week 4). Superscript a indicates significant differences from saline controls ($p < 0.05$).

Effects of PTH₁₋₃₄ treatment regimen on gene expression during femoral allograft healing

To further elucidate the mechanism responsible for delayed PTH₁₋₃₄ effects on allograft healing, we performed a time course experiment to assess changes in mRNA expression of markers of chondrocytes, angiogenesis, osteoblasts, and osteoclasts over 28 days of healing (Fig. 5). The results of these experiments corroborated several of the histologic findings. First, only the immediate PTH₁₋₃₄ treatment had significant effects on chondrogenic (*colla1*) and hypertrophic (*col1a1*) gene expression that disappeared by 10 to 14 days, which is consistent with the small increases in cartilage area at 2 weeks and the absence of persistent cartilage at 4 weeks. Immediate PTH₁₋₃₄ treatment also increased the expression of *mmp9* and *vegfa*, which are associated with cartilage removal and angiogenesis during fracture healing, at 14 days postsurgery.^(17,21) Most importantly, we found that all regimens of high dose PTH₁₋₃₄ significantly ($p < 0.05$) increased markers of bone formation (*colla1*, and *oc*) on days 21 and 28 postsurgery, which is consistent with the sustained bone formation at 4 weeks (Supplemental Figs. 2 and 3).

Discussion

Despite its clinical effectiveness in the treatment of osteoporosis,^(22,23) reports of clinical off-label use to resolve fibrotic nonunion fractures,⁽²⁴⁾ and a growing body of preclinical data from various animal studies that raised expectations of its efficacy in accelerating bone repair,^(5-7,9,10,25-28) parathyroid hormone (PTH) therapy has yet to be widely clinically adopted for the management of nonoperative fractures or the more challenging cases of fibrotic nonunions. There are several reasons that contribute to the slow translation of PTH to the clinical or surgical management these indications. First, there have been safety concerns regarding the range of effective PTH₁₋₃₄ doses in the animal studies of fracture repair (10–200 µg/kg/day), which correspond to tens or hundreds of multiples of the FDA approved doses of 20 or 40 µg/day (0.25 to 0.5 µg/kg/day for an 80-kg person) for treatment of osteoporosis. The high doses, in combination with long treatments, have been linked to high incidence of osteosarcomas in rats,^(23,29) which despite evidence of safety from surveillance studies of patients

previously treated with PTH₁₋₃₄, led the FDA to approve the drug with strong recommendations to limit its prescription to women and men with substantial osteoporosis and susceptibility to fracture, and placed a black box exclusion warning for patients at high risk for osteosarcoma.⁽²³⁾ This regulatory barrier is compounded by the absence of long-term patent protection, and hence, the lack of commercial incentive to perform expensive safety and efficacy clinical trials for challenging fractures.⁽²³⁾ Moreover, there is no consensus on the outcome variables that could be used to definitively establish efficacy in human patients.⁽³⁰⁾ A case in point is a recent study that indicated that the clinical value of PTH (teriparatide) treatment in distal radial fractures (assessed by the time to healing based on radiographic evidence of cortical union, patient-related wrist evaluation score, and early callus formation) was limited to marginal acceleration of healing that did not provide strong cost-risk-benefit justification.^(13,30) The inconclusive findings of this human trial in contrast to the efficacy evidence in animal studies underscore its limitations: (1) the lack of validated thresholds of bone healing based on non- or minimally invasive assessment, and (2) the fact that these fractures still healed with placebo in just weeks, which makes establishing the case for treatment efficacy difficult. Finally, there remain many unanswered questions about the appropriate timing of commencement and withdrawal of the treatment, which have not been thoroughly investigated in preclinical studies. Hence, the motivation for the current study was to address the latter questions of dose, timing, and duration of treatment, in hopes of identifying in a preclinical model a short treatment window that could help abate the more serious concerns about osteosarcoma risk in nonlife threatening indications such as fracture repair, which could then incentivize definitive large animal studies and clinical trials of safety and efficacy.

Because allografts are typically complicated by nonunion and therefore are more challenging than osteotomy or closed fracture models, we elected to investigate PTH₁₋₃₄ treatment regimens in a previously established mouse femoral reconstruction model.^(11,14,20) We have recently shown in this model that daily PTH₁₋₃₄ (40 µg/kg/day) commencing immediately after devitalized allograft reconstruction for 6 weeks induced trabeculated bone callus formation and remarkable graft-host integration, which were correlated with more than doubling of the biomechanical parameters of torsional rigidity and yield

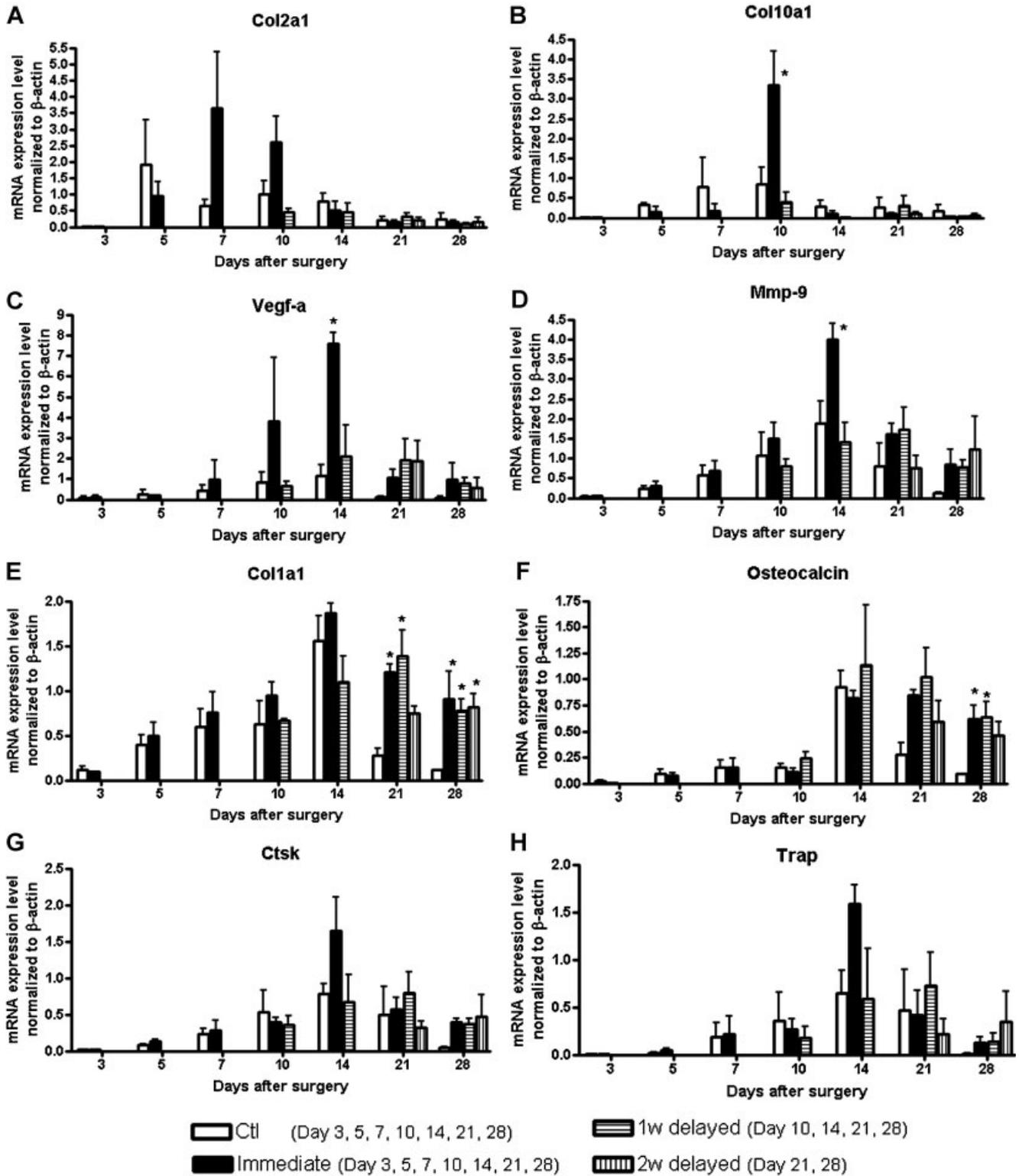


Fig. 5. Analysis of mRNA expression levels at the allograft–host bone junctions over time up to 28 days postsurgery. Gene expression was quantified using real-time RT-PCR for selected markers of chondrogenesis (*col2a1*), hypertrophy, and remodeling (*col10a1*, *vegfa*, and *mmp9*), osteogenesis (*col1a1* and *oc*), and osteoclastogenesis (*ctsk* and *trap*). Bar plots represent means and SEM ($n = 3$ per time point). Asterisks indicate significant differences versus controls ($p < 0.05$).

torque compared with saline treated controls.⁽¹⁵⁾ In the current study, we first asked whether lower doses of PTH_{1–34} could be as effective as higher dose in enhancing allograft osseointegration and biomechanics. Consistent with several previous studies in

rodent models of fracture repair,^(5–7,9,10,25–28) our findings demonstrated that only the high daily dose (40 $\mu\text{g}/\text{kg}/\text{day}$) showed remarkable effects on torsional properties of grafted femurs when administered for 9 weeks, whereas the lower doses

of 0.4 and 4 $\mu\text{g}/\text{kg}/\text{day}$ did not provide any benefits compared with controls. Rodent models typically report the use of doses as high as 10 to 200 $\mu\text{g}/\text{kg}/\text{day}$. Although the difference between the effective clinical dose for osteoporosis and the much higher doses for rodent models of fracture repair are thought to arise from differences in metabolism and clearance of PTH, and typically require treatment periods amounting to >50% of the animal life span,⁽³¹⁾ conclusive statements about the effectiveness of lower dose might still require investigation in larger animal species that might better approximate the metabolism of the drug in humans, and a clinically relevant treatment window.

Our studies showed that immediate PTH treatment substantially improved early cartilaginous template formation in the fracture callus and subsequent mineralization, leading to improved healing at the host–allograft junction. This finding is supported by several previous reports that describe the main effects of intermittent PTH treatment on fracture repair to be through enhancement of the early proliferative response of chondroprogenitor and osteoprogenitor cells.^(9,10,31)

We also observed that short-term treatments (2 weeks), commencing immediately or with a 1- or 2-week delay after reconstruction, were just as effective in enhancing the biomechanical properties at 9 weeks as the continuous daily treatment for 9 weeks. This data suggests that short-term treatment during the first 4 weeks after surgery is sufficient to improve graft–host union from fibrous union to osseous union and that establishing osseous union is a primary mechanism of PTH_{1–34}-induced increase in mechanical strength of grafted femur. These observations have great clinical relevance. The effectiveness of the short treatments could abate concerns related to osteosarcoma risk with prolonged treatment. Furthermore, given that PTH therapy is delivered via inconvenient subcutaneous injections and is very expensive, short treatment could enhance patient compliance and balance cost–benefit concerns. Moreover, because a likely clinical scenario that could benefit from PTH therapy might involve patients in whom bone repair may be delayed,⁽³¹⁾ the effectiveness of the delayed treatment in our murine model should encourage further investigation in larger species.

The observation that 1- or 2-week delayed short-term treatment was as effective as immediate treatment suggests that PTH_{1–34} might have different actions depending on the overlap with the different stages of healing and the cells involved. Our results corroborate previous findings that suggest that immediate treatment with PTH_{1–34} might have multiple effects that include suppressing inflammation upon surgery,⁽³²⁾ early stimulation of proliferation of mesenchymal and periosteal stem cells,⁽¹⁰⁾ and enhanced early differentiation of chondroprogenitors leading to robust “endochondral ossification” bone repair,^(9,31) which is consistent with our observations of increased callus size in association with PTH_{1–34} treatments. On the other hand, 1- or 2-week delayed PTH therapy in which the treatment missed part or all of the endochondral ossification phase still induced persistent osteoblastic and osteoclastic activity up to 4 weeks. This was manifested by remarkable increases in *colla1* and *oc* gene expression at day 21, which began to decline by day 28. Interestingly, the 4-week delay in the treatment could not

improve the biomechanical properties of the allografted femurs compared with controls. These findings suggest that bone repair could still be accomplished, even if treatment is delayed, via intramembranous ossification, and further suggest that the first 3 weeks could be the critical window for PTH_{1–34} therapy. Given that bone formation peaks at around 2 weeks after surgery and is downregulated by 4 weeks after surgery in control mice that underwent femoral allograft surgery, PTH_{1–34} therapy would be effective as long as active bone formation sustains after surgery but becomes ineffective after repair reaction is attenuated. Some clinical case reports of off-label use of PTH_{1–34} demonstrated that patients with prolonged fracture nonunion would benefit from anabolic therapy months to years after fracture,^(11,33,34) to our knowledge, however, there are no definitive data as to by when after surgery PTH_{1–34} would be effective for bone repair. Therefore, it should be tested in larger animal models or clinically how long after surgery PTH_{1–34} could be expected to show positive effect on bone repair. Although we failed to observe significant differences in osteoclast marker gene expression (*ctsk* and *trap*) during this critical phase of allograft healing, this was not surprising, as the significant PTH_{1–34} effects on osteoclasts were on their location rather than total numbers. As the osteoclasts on the graft surface are primarily involved in generating a new marrow space between the necrotic and new bone, while the osteoclasts in the callus are remodeling the osteoid into lamellar bone, this finding suggests that PTH_{1–34} effects are pertinent for callus remodeling, which was less of a factor in delayed treatment. Thus, it is unlikely that the mechanism responsible for delayed PTH_{1–34} treatment effects on structural allografting involves increased bone resorption. Future studies should address the effects of PTH_{1–34} on the different cellular compartments involved in the repair and remodeling of the allograft.

Our findings provide strong evidence in support of the efficacy of delayed, short PTH_{1–34} treatment in a mouse model of challenging bone repair. A recent study investigated the effects of different PTH administration regimes applied at different stages of fracture healing in an osteoporotic (ovariectomized or Ovx) rat model of tibial osteotomy healing,⁽³⁵⁾ and similarly reported that the treatment improved fracture repair compared with untreated controls when PTH was administered either immediately or 7 days postosteotomy, independent of the administration frequency, but not after a 14-day delay. Collectively, our findings and others' should motivate further investigation of the efficacy of PTH treatment in challenging bone repair scenarios in larger species and clinical studies.

Disclosures

All authors state that they have no conflicts of interest.

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