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Teriparatide Therapy Enhances Devitalized Femoral Allograft Osseointegration and Biomechanics in a Murine Model

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Abstract

Despite the remarkable healing potential of long bone fractures, traumatic injuries that result in critical defects require challenging reconstructive limb sparing surgery. While devitalized allografts are the gold standard for these procedures, they are prone to failure due to their limited osseointegration with the host. Thus, the quest for adjuvants to enhance allograft healing remains a priority for this unmet clinical need. To address this, we investigated the effects of daily systemic injections of 40 µg/kg teriparatide (recombinant human parathyroid hormone) on the healing of devitalized allografts used to reconstruct critical femoral defects (4 mm) in C57Bl/6 mice. The femurs were evaluated at 4 and 6 weeks using micro CT, histology, and torsion testing. Our findings demonstrated that teriparatide induced prolonged cartilage formation at the graft-host junction at 4 weeks, which led to enhanced trabeculated bone callus formation and remarkable graft-host integration at 6-weeks. Moreover, we observed a significant 2-fold increase in normalized callus volume (1.04 ± 0.3 vs. 0.54 ± 0.14 mm³/mm; $p < 0.005$), and Union Ratio (0.28 ± 0.07 vs. 0.13 ± 0.09 ; $p < 0.005$), compared to saline treated controls at 6-weeks. Teriparatide treatment significantly increased the torsional rigidity (585 ± 408 versus 1175 ± 311 N.mm²) and yield torque (6.8 ± 5.5 versus 10.5 ± 4.2 N.mm) compared to controls. Interestingly, the Union Ratio correlated significantly with the yield torque and torsional rigidity ($R^2 = 0.59$ and $R^2 = 0.77$, $p < 0.001$, respectively). These results illustrate the remarkable potential of teriparatide as an adjuvant therapy for allograft repair in a mouse model of massive femoral defect reconstruction, and warrant further investigation in a larger animal model at longer time intervals to justify future clinical trials for PTH therapy in limb sparing reconstructive procedures.

Keywords

Bone; Allograft; PTH; Biomechanics; Micro-Computed Tomography

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Introduction

While long bone has a remarkable healing capacity, it has limited regenerative potential. Indeed, traumatic injuries that lead to critical defects (>3 cm) are a major surgical challenge. Both amputation and limb salvage procedures have high rates of self-reported disability (40% to 50%) and continue to worsen over time [1,2]. Additionally, the financial costs associated with these poor outcomes are a major burden on our health care system. A recent study in 8 major U.S. health centers calculated the costs of managing these injuries for 2 years, and also estimated their lifetime costs [3]. The 2-year average costs for limb salvage were \$81,316 versus \$91,106 for amputation. The lifetime costs were \$163,282 and \$509,275 per patient, respectively. While these clinical complications and high costs of care have been well documented in the civilian population, it is important to also note that 26% of recent combat nonfatal casualties are fractures, and 82% of these fractures are open and require limb salvage [4]. At this time, massive allografts are the dominant clinical option for limb reconstruction and salvage, although they are prone to structural failure, which limits their longevity due to their inability to incorporate and remodel [5]. Thus, the quest for a practical adjuvant therapy for standard limb salvage with massive allografts is an unmet clinical need.

Although recombinant human BMP2 (Infuse™) and various cell-based therapies are being investigated for this purpose [6], their intraoperative application approach involves many technical challenges that have produced only modest clinical benefits to date. The emergence of parathyroid hormone (teriparatide) as the first and only systemically administered anabolic therapy holds tremendous promise as an adjuvant for bone healing in challenging contexts. However, despite the extensive peer reviewed literature concluding that different forms of PTH are effective in small animal models of fracture healing [7–12], and anecdotal clinical case reports of off label use of teriparatide to heal delayed and non-unions [13], the effects of PTH on massive allografts for limb salvage have not been evaluated.

In this study, we hypothesized that intermittent teriparatide (recombinant human PTH(1,34)) administration improves allograft-to-host union and, by corollary bone strength and rigidity following allograft reconstruction of massive mouse femoral defects. To test this hypothesis, we used a preclinical mouse model of femoral allograft reconstruction, which we previously developed [14] to test therapeutic approaches that can potentially enhance osseointegration of massive bone allografts [15,16]. Furthermore, we employed a novel quantitative measure of allograft union that we previously described [17] to correlate the teriparatide induced allograft-host union with the biomechanical properties of the grafted femurs [18].

Materials & Methods

Preparation of Decellularized Allografts

As previously described [19], devitalized femoral allografts were prepared from femurs of 10 week old, female, C57Bl/6 donor mice. The femurs were harvested aseptically, and scraped of periosteal tissue with a scalpel, cut to 4 mm in length, and flushed of bone marrow using saline from a syringe. The allografts were devitalized by bathing in 70% ethanol for 3 hours, and then washing in phosphate buffered saline before storing at -70°C for 1 week.

Femoral Allograft Reconstruction Surgery

Animal surgery and care protocols were performed in accordance with the regulations of the University of Rochester's Committee on Animal Resources as previously described [14]. Briefly, allografts were implanted into a 4 mm defect created in the mid-diaphysis of the left

femur of the recipient mice (10 week old, female, C57Bl/6 strain) using a 10mm diameter, 0.15mm thick diamond-sintered rotary saw (Small Parts, Inc, Miramar, FL) and secured in place with an intramedullary pin. One week after surgery, daily subcutaneous injections of 40µg/kg teriparatide (PTH) (Forteo™, Eli Lilly and Co., Indianapolis, IN) were initiated in the experimental group, whereas the control group received placebo saline injections. The one-week delayed treatment was meant to simulate the establishment of fibrous non-union, a clinical scenario in which teriparatide might be used off-label to treat nonhealing fractures that develop persistent fibrous non-unions [13]. The animals were sacrificed at either 4 or 6 weeks after surgery. The allografts and the unoperated contralateral femurs were characterized using as per the experimental design described in Table 1.

Micro Computed Tomography Imaging of Bone Formation and Union

Upon sacrifice at 4 or 6 weeks, the femurs were disarticulated at the hip and knee joints and the intramedullary pins were carefully removed. Specimens underwent one freeze-thaw cycle (−20°C) before micro computed tomography (micro CT) imaging. The specimens were scanned at 12.5 microns isotropic resolution using the VivaCT 40 (Scanco Medical AG, Bassersdorf, Switzerland). From these 3D images, the graft and callus bone volumes (BV_{Graft} , BV_{Callus}) were measured by manual segmentation, followed by standardized thresholding at a grayscale corresponding to 750 mgHA/cm³ based on a phantom of known HA concentrations. The cross sectional polar moments of inertia (PMI) was calculated for each slice throughout the grafted region by integration about the area centroid as previously described [18]. Briefly, numerical integration of mineralized pixels (based on a threshold corresponding to 750 mg HA) was performed, based on the equation $PMI = \int r^2 dA$ where dA is the elemental area of each mineralized pixel, and r is the radial distance to the element dA from the cross-section centroid. The average, minimum, and maximum cross-sectional polar moments of inertia (PMI_{Ave} , PMI_{Min} , and PMI_{Max}) were computed for each specimen.

The Union Ratio was also calculated as recently described [17]. The Union Ratio, a measure of allograft osseointegration, is based on the minimum graft surface area fraction upon which mineralized callus had formed. Briefly, a semi-automated Matlab algorithm morphs manually drawn contours of the allograft on micro-CT slices and snaps them to the surface of the graft using edge detection. This algorithm then attempts to detect a non-mineralized gap at the graft edge, which indicates a region of graft non-union and if found, the contour dilates into that space. If no gap is found, the contour does not dilate and remains in contact with the graft's edge, which indicates a region of union. Integrating the 2D contours along the entire length into a 3D shell allows for measurement of union surface area along the length of the graft. The union area in each half (proximal and distal) of the graft is computed separately and the lesser fraction of union area to total graft surface area is given as the Union Ratio for that specimen [17]. The formation of a contiguous mineralized callus bridge that integrates one end of the graft to the other was also evaluated from serial sagittal micro CT sections.

Histology

After micro CT imaging, specimens were processed for histology. Mid-sagittal sections were stained with alcian blue, hematoxylin, eosin and orange G. Osteoclast number and activity were evaluated from sections stained for tartrate resistant acid phosphatase (TRAP) by counting the number of TRAP positive cells on the bone surface, as previously described [14].

Biomechanical Testing and Failure Mode Analysis

Immediately following micro CT imaging of bone, the torsional biomechanical properties of the grafted femurs were determined as previously described [18] using an EnduraTec TestBench™ system (200 N.mm torque cell; Bose Corporation, Minnetonka, MN) at a rate of 1°/sec. Yield torque (T_{Yield}), ultimate torque (T_{Ult}), torsional rigidity (TR), toughness (or work to failure) and the twist at ultimate torque were determined for each specimen. After torsion testing, the specimens were x-rayed to analyze the mode of failure as previously described [18]. Briefly, failures occurring only at the graft-host interface where the graft simply pulled out from the surrounding callus were considered failures due to poor union, and labeled “pre-union”. Failures involving fractures within the graft and/or host bone indicate that there was some bonding of the host on the graft thus were not considered simple non-unions. These were categorized into “early union” if the fracture involved the graft-host interface and “mature union” if the failure involved a spiral fracture of the graft midsubstance, respectively.

Statistical Analysis

Following an F-test of the homogeneity of the variances, independent student t-tests were used to determine the significance ($p < 0.05$) of teriparatide effects on bone formation and union, biomechanical properties, and vascular parameters compared to saline controls. As previously described [18], univariate regression analysis was performed to correlate independent micro CT derived parameters with biomechanical properties (ultimate torque, yield torque, and torsional rigidity). In addition, multivariate regression analysis with stepwise selection was performed to optimize the combination of significant ($p < 0.05$) micro CT variables that correlated with the torsional mechanical properties in a general linear model [18]. SAS 9.1 (SAS Institute Inc., Cary, NC) was used for the statistical correlations.

Results

Teriparatide Therapy Increases Bone Formation and Osseointegration of Femoral Allografts

We evaluated the effects of teriparatide-induced bone formation and osseointegration on femoral allograft healing by performing micro-CT with subsequent histology analyses on grafted femurs at 4 and 6-weeks post-op (Figure 1). At 4 weeks, saline treated allografts already had a completely remodeled new bone callus without cartilage. However, they also contained fibrotic tissue that filled the medullary canal and surrounded the periosteal surface, which appeared as a gap between the allograft and new callus bone on 2D micro-CT (Fig. 1a–c). In contrast, the callus of femoral allografts from teriparatide treated mice at 4-weeks had a remarkable amount of persistent cartilage and bone marrow that was filled with woven and trabecular bone. Moreover, most of the fibrotic tissue that was observed in the saline group was replaced by contiguous cartilage between the allograft periosteal surface and the new bone callus, which also appeared as a soft-tissue gap on 2D micro-CT (Fig. 1d–f). At 6-weeks the callus of saline treated grafted femurs had remodeled further to cortical bone filled with cellular bone marrow with little trabecular bone (Fig. 2g–i). The lack of osseointegration between the remodeled callus and the cortical allograft surfaces were also apparent from the histology. In contrast, the remodeled callus of teriparatide treated allografts remained filled with dense trabecular bone that occupied most of the callus area around the allograft (Fig. 2j–l). Remarkably, both 2D micro-CT and histology demonstrate that drug-induced trabecular bone also penetrated the medullary canal of the allograft, and that the copious trabecular bone was contiguous with the allograft on both periosteal and endosteal surfaces. Collectively, these data suggest that teriparatide therapy increases osseointegration during allograft healing by significantly increasing the amount of new trabecular bone that forms and is connected to the allograft.

Volumetric micro CT imaging revealed that teriparatide induced larger and more densely packed, trabecular bone formation around the allograft and in the intramedullary marrow space that was not observed in control specimens. Compared to controls, teriparatide treatment significantly increased the volume of the mineralized callus forming on the periosteal graft surface (BV_{Callus}) and in the intramedullary space (BV_{Intramed}) by 93% and 217%, respectively (Table 2). This enhanced callus formation resulted in a significant increase in PMI_{Ave} and PMI_{Max} by 38% and 26%, respectively, predominately due to a uniform increase in cross sectional area and bone volume fraction of the callus, rather than a dramatic change in the diameter of the callus. Teriparatide treatment significantly increased total callus mineral content over controls by 67%, despite a 14% decrease in bone mineral density. On the other hand, there was no teriparatide related difference in allograft bone volume compared to controls, which suggests that there was no increase in graft resorption with the treatment at 6-weeks. This was confirmed by histology, which revealed no difference in the number of osteoclasts or resorption surfaces on the allografts (data not shown). However, further analysis using dynamic bone labeling (tetracycline or calcein injections) can provide definitive conclusions regarding the remodeling of the allografts or the lack thereof. More importantly, teriparatide treatment significantly increased the Union Ratio by 76% over controls (Figure 2, Table 2). Interestingly, teriparatide treatment also resulted in significant increases in the cortical thickness, cross-sectional area, and PMI of the unoperated contralateral femurs (Table 3).

Teriparatide Therapy Increases the Biomechanical Properties of Femoral Allografts

In order to determine if the teriparatide-induced histologic and radiographic increases in bone translated into improved mechanical properties we performed torsion testing on the grafted and unoperated contralateral femurs at 6 weeks, immediately after micro CT imaging. Teriparatide treatment significantly increased the grafted femur yield torque (T_{yield}) and torsional rigidity (TR), and decreased the twist angle at failure, reflecting a more brittle behavior compared to controls (Figure 3). The torsional rigidity of grafted femurs treated with teriparatide was double the controls ($p < 0.05$), and was not significantly different from the treated or untreated contralateral femurs. The yield torque in teriparatide treated grafted femurs was also significantly 72% greater than controls ($p < 0.05$), but there was no significant difference in the ultimate torque, despite a 23% increase in the teriparatide treated grafted femurs. Saline treated grafted femurs failed in a much more ductile manner than those with PTH treatment as indicated by the maximum twist at failure (Figure 3). X-ray scoring of the mode of failure was assessed using Fisher's Exact Test and yielded no significant treatment related differences, although only 23% of the control specimens exhibited early or mature union (involving graft-host interface or graft midsubstance, respectively), compared to 42% of the teriparatide treated allografts.

Correlations Between Micro CT Parameters and Torsional Properties

A critical goal towards the translation of teriparatide therapy for allograft healing in humans is the development of minimally invasive outcome measures that correlate with functional biomechanics. To this end we investigated associations between micro CT derived measures of functional healing and the biomechanical outcomes using univariate and multivariate linear regression analyses. As a single variable, the Union Ratio correlated significantly with subfailure (yield torque and torsional rigidity) and failure (ultimate torque) biomechanical properties (Table 4). However, while the Union Ratio was the best single variable predictor for the torsional rigidity ($R^2 = 0.77$, $p < 0.001$) and yield torque ($R^2 = 0.59$, $p < 0.001$), the best univariate predictor for the ultimate torque was PMI_{Min} ($R^2 = 0.57$, $p < 0.001$). Interestingly, when examining these correlations, we observed a "functional healing quadrant" wherein 86% of the teriparatide-treated specimens reached a union ratio of 0.2 (arbitrary threshold) while at the same time achieving torsional rigidity values greater than the lower bound of the

99% confidence interval of normal unoperated femurs' torsional rigidity (shaded quadrant, Figure 4a). In addition, 64% of the teriparatide-treated specimens reached a union ratio of 0.2 while at the same time achieving yield torque values that exceeded the lower bound of the 99% confidence interval of normal unoperated femurs' yield strength (shaded quadrant, Figure 4b). In comparison, the percentages of placebo-treated specimens that achieved this functional healing criterion were only 14% and 7% for the torsional rigidity and yield strength, respectively. Given that several other micro CT parameters correlated significantly with torsional properties (Table 4), we performed stepwise multiple regression analysis as previously described [17]. The torsional rigidity correlated significantly with a linear combination of Union Ratio and PMI_{Min} (Figure 4c). Similarly, the yield torque correlated significantly with a linear combination of Union Ratio and PMI_{Min} , and PMI_{Ave} (Figure 4d).

Discussion

Large structural allografts are the biomaterial of choice for reconstructing massive long bone defects that might otherwise require prosthetics or amputation. Despite their abundant availability through tissue banks, processed allografts, which are typically devitalized, experience major complications that affect their functional longevity, including non-unions (27–34%) and graft fractures (24–27%) [5,20]. The ability of the devitalized allograft to repair micro-damage and establish union with the host is limited due to the lack of osteogenic, angiogenic, and remodeling capabilities [15,19,21]. It is therefore important to design treatment strategies that enhance the functional longevity of processed allografts by addressing the aforementioned biological limitations. Strategies that include cell grafting and localized growth factor or gene delivery have been investigated in preclinical models of massive defects with encouraging results [15,16,22–25], but to date have not been widely implemented clinically. On the other hand, strategies that involve endocrine hormone treatment (*e.g.* parathyroid hormone or PTH), which have shown efficacy in preclinical animal models of fracture repair and are arguably easier to implement clinically, have not been investigated in the context of enhancing structural allograft osseointegration and healing. To address this, the current study investigated the efficacy of teriparatide on functional integration of devitalized allografts in a mouse model of femoral reconstruction.

Our findings demonstrated that daily systemic injection of teriparatide (40 μ g/kg body weight) induced persistent cartilage and enhanced trabecular bone formation at 4 weeks (Figure 1), which led to remarkable graft-host integration at 6-weeks (Figure 2). Moreover, we observed a significant 2-fold increase in normalized callus volume (1.04 ± 0.3 vs. 0.54 ± 0.14 mm³/mm, $p < 0.005$), and Union Ratio (0.28 ± 0.07 vs. 0.13 ± 0.09 ; $p < 0.005$), compared to saline treated controls at 6-weeks. Most importantly, the Union Ratio correlated significantly with the yield torque and torsional rigidity ($R^2 = 0.59$ and $R^2 = 0.77$, $p < 0.001$, respectively).

The effects of PTH therapy on bone turnover and osteoporotic fractures have been investigated since 1980 [26–28]. Numerous preclinical studies have since shown that multiple forms of PTH are capable of accelerating the rate of fracture healing [8–10,29–33]. Most of these studies used closed (Einhorn) fracture models whose healing is not typically complicated by nonunion. The combination of systematic parathyroid hormone and a local parathyroid hormone gene therapy in non-union osteotomy models has also shown promising efficacy in inducing bone regeneration across a critical size defect [8,34]. While only approved as a prophylactic treatment in osteoporotic patients with high fracture risk, off-label clinical use of teriparatide in patients with delayed nonunion fractures has been recently reported with encouraging preliminary outcomes, which led to clinical trials on the use of teriparatide to accelerate fracture healing [13,35].

Teriparatide effects on bone grafts have only recently been reported. Intermittent teriparatide treatment has been shown to increase the strength and bone mineral content in a rat model of freshly harvested, vascularized long bone allograft, either alone or when followed by treatment with the bisphosphonate zoledronic acid [36]. It has also been reported that intermittent administration of teriparatide accelerates rat spinal arthrodesis after autologous bone grafting [37]. To the best of our knowledge, our work is the first to investigate the effects of intermittent PTH or teriparatide treatment on critical-size cortical defects reconstructed with devitalized allografts.

Our results are remarkably similar to the PTH effects reported in rodent models of fracture repair and autografts, despite the biological and biomechanical differences in devitalized cortical allograft healing which we previously reported [14,18]. However, there is a significant discrepancy between the doses reported in the literature in preclinical animal models (including ours) and the clinical doses in indicated and off-label uses. For example, animal models typically report the use of doses as high as 2.5 to 200 micrograms/kg/day which are 10- to 1000-fold greater than the recommended clinical dose in humans (20 micrograms per day total, which in a 50 to 80kg person translates to 0.25 to 0.4 micrograms/kg/day). Interestingly, studies that addressed dose-dependent effects of PTH in rodents failed to show an effect at the lower doses [10,12], which has been attributed to differences in the hormone metabolism between species [11]. An alternative explanation is that the fracture models used in these studies are not critical defects or nonunion arthrodesis, and typically heal on their own, which confounds any effects a low dose of the drug might exert. Therefore, the appropriate clinically relevant doses of the drug in a challenging bone healing context, such as devitalized allografts, warrants further investigation.

In addition, the timing of the commencement and withdrawal of the treatment are also important. In the scenario of nonunion arthrodesis, the treatment is likely to be delayed following the radiographic diagnosis of nonunion. However, in a scheduled allograft reconstruction, the treatment can be initiated immediately at the time of surgery. Whether immediate treatment is superior to delayed treatment has not been determined and warrants formal investigation. The therapeutic PTH treatment window is important, as it will likely dictate the cell populations that respond to the drug during the multiple stages of the bone healing process. In osteoporosis, PTH has a direct effect on osteoblasts and activates their proliferation and osteocalcin production while suppressing apoptosis [38,39], without affecting osteoclast activity [38]. On the other hand, PTH has been reported to activate the differentiation of bone marrow stromal cells (BMSCs) [40], and bone lining cells in vivo, without affecting proliferation [41]. In addition to these effects on intact bone and osteoprecursor cells, PTH has been shown to enhance fracture healing by increasing proliferation of chondroprogenitors which leads to a bigger cartilage template formation [10,42], ultimately leading to an increase in callus volume and bone mineral content via accelerated chondrocyte hypertrophy [42]. Consistent with these observations, daily teriparatide injections, which were initiated one-week post surgery in our model of allograft reconstruction, resulted in persistent cartilage formation at 4 weeks, which led to improved graft-host integration at 6-weeks via a remarkable periosteal and endosteal trabeculated bone callus. Our study, however, has limitations in that it only evaluated a single regimen of teriparatide at a single time point in young animals. Future studies are warranted to investigate teriparatide dose and treatment timing and duration effects on allograft healing at longer time points, and following various intervals of treatment withdrawal in both young and aging animal models.

One of the most important findings of the current work was demonstrating that the Union Ratio, a micro-CT derived biometric of osseointegration between the allograft and host bone [17], was a significant predictive variable of the effects of teriparatide on the subfailure

biomechanical properties of the allografts. More interestingly, we observed that a minimum Union Ratio threshold must be achieved to radiographically determine functional healing as defined by achieving normal torsional rigidity and yield strength with 99% confidence. Whether this Union Ratio threshold value of 0.2 would scale up in larger animals and human patients would have to be verified in future studies. However, despite the strong univariate correlations, there were several outliers that either achieved functional healing (as defined by torsional properties) without achieving the Union Ratio threshold, or that failed to achieve functional healing despite achieving Union Ratio values ≥ 0.2 . These outliers underscore one of the major limitations of the Union Ratio as a measure of functional healing. Specifically, the Union Ratio is a biometric measuring the ratio of connected graft area to the host based on a binary determination of the voxels as either bone or not bone that does not account for any gradation in the mineral density of the union tissue. Future efforts will be dedicated to developing a mineral density-weighted Union Ratio to account for the mineralized state of the union area, which we hypothesize will improve correlations with the biomechanical properties, and eliminate the outliers in radiographically predicting functional healing.

In conclusion, our data illustrate the remarkable potential of teriparatide (PTH) therapy in enhancing allograft healing and integration with the host in a mouse model of massive femoral defect reconstruction. These results should motivate further investigation in a larger animal model to justify future clinical trials for this indication.

Research Highlights

- Teriparatide protracts persistent cartilage formation leading to trabeculated bone callus
- Teriparatide enhances osseointegration and biomechanics of devitalized femoral allografts
- Union Ratio measure of osseointegration correlates significantly with torsional biomechanics
- A functional healing criterion could be defined using the non-invasive Union Ratio biometric

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Figure 1. Daily teriparatide treatment prolongs persistent cartilage formation at 4 weeks leading to increased trabeculated bone callus on the periosteal and endosteal surfaces of the allograft at 6 weeks

Representative sagittal sections of grafted femurs treated with saline (*a–c* and *g–i*) and 40 μ g/kg teriparatide (*d–f* and *j–l*) at 4 and 6 weeks, respectively. The left panels (*a, d, g, j*) represent sagittal micro-CT radiographs of the proximal graft-host interface, while the middle panels (*b, e, h, k*) show the corresponding histological sections stained with Orange G and alcian blue. The right panels (*c, f, i, l*) show magnified views of the boxed region of interest. Asterisks label the proximal end of the graft segment. IM indicates the intramedullary space and P indicates the periosteal callus.

Figure 2. Daily teriparatide treatment enhances devitalized allograft osseointegration with the host bone, and increases the Union Ratio via extensive trabeculated bone callus formation on the periosteal and endosteal surfaces of the allograft at 6 weeks

Representative micro-CT volumetric rendering of the grafted femurs treated with saline (*a–b*) and 40 mg/kg daily teriparatide injections for 6 weeks (*e–f*). The allograft is highlighted by the yellow tint in the sagittal sections (*b and f*), where the proximal ends of the allograft are identified by the yellow arrows. Of note is the gap between the callus and the allograft in the saline treated specimens (*c*) compared to the intimate trabeculated bone formation on the endosteal and periosteal allograft surface (*g*). The corresponding union area maps and union ratio numerical values are shown for the saline (*d*) and teriparatide (*h*) treated grafts, wherein red pixels indicate surface areas where the graft is connected to the host. Note that union areas on the periosteal and endosteal surfaces, and the ends of the graft were all accounted for. The proximal and distal halves of the graft were evaluated separately, and the lowest value of the union area normalized by the surface area was reported as the Union Ratio (Table 2), as previously described [17].

Figure 3. Daily teriparatide treatment improves the biomechanical torsional properties of grafted femurs

Grafted and unoperated contralateral femurs were retrieved upon animal sacrifice at 6 weeks and tested in torsion at 1°/sec to determine the ultimate (a) and torque (b) as well as the torsional rigidity (c) and twist at failure (d). Data presented as mean ± standard deviation. Asterisk indicates significant differences due to the teriparatide treatment between the grafted femurs.

Figure 4. Micro CT-Derived parameters predict functional healing defined by torsional properties

The Union Ratio correlates significantly with (a) the torsional rigidity and (b) the yield torque ($R^2 = 0.77$ and 0.59 ; respectively). Multivariate correlations of the torsional rigidity (c) and yield torque (d) demonstrate that linear combinations of the Union Ratio and the Polar Moment of Inertia correlate significantly with torsional properties ($R^2 = 0.85$ and 0.81 , respectively). Solid black lines in a–d represent the correlations trend lines. Solid and dashed red lines in a and b represent the mean and the upper and lower bounds of the 99% confidence interval (CI) of normal unoperated femur values, respectively. Shaded area represents the “functional healing” quadrant in which grafted femurs achieved both a Union Ratio of 0.2 (arbitrary threshold) and functional healing (defined as reaching the lower bound of the 99% CI of the torsional rigidity and yield torque, respectively). Note that 86% of the teriparatide treated femurs fell within the functional healing quadrant for the torsional rigidity compared to only 14% of the saline treated specimens. Similarly, 64% of the teriparatide treated specimens fell within the yield torque functional healing quadrant compared to only 7% of the saline treated controls.

Table 1

Experimental Groups, Response Measures, and Sample Sizes.

Group/Femur	Treatment	Analysis	Time Point	Sample Size
1 Allograft	Saline (Control)	μCT + Histology	4 weeks	n=4
2 Allograft	Teriparatide (40μg/kg/day)	μCT + Histology	4 weeks	n=4
3 Allograft	Saline (Control)	μCT + Histology	6 weeks	n=4
4 Allograft	Teriparatide (40μg/kg/day)	μCT + Histology	6 weeks	n=4
5 Allograft	Saline (Control)	μCT + Torsional Testing	6 weeks	n=14
6 Allograft	Teriparatide (40μg/kg/day)	μCT + Torsional Testing	6 weeks	n=14
7 Unoperated	Saline (Control)	μCT + Torsional Testing	6 weeks	n=14
8 Unoperated	Teriparatide (40μg/kg/day)	μCT + Torsional Testing	6 weeks	n=14

Table 2

Micro CT-derived Parameters of the grafted femurs at 6 weeks, presented as mean (standard deviation). P values indicate significant differences between the teriparatide treated samples and the saline treated controls, while NS indicates no significant differences.

	Saline	Teriparatide	
PMI _{Min} (mm ⁴)	0.41 (0.11)	0.51 (0.26)	NS
PMI _{Ave} (mm ⁴)	0.84 (0.14)	1.16 (0.32)	p<0.005
PMI _{Max} (mm ⁴)	1.72 (0.59)	2.17 (0.46)	p<0.05
BV _{Graft} (mm ³ /mm)	0.84 (0.066)	0.82 (0.067)	NS
BV _{Callus} (mm ³ /mm)	0.54 (0.14)	1.04 (0.3)	p<0.005
BV _{Intramed} (mm ³ /mm)	0.063 (0.042)	0.2 (0.081)	p<0.005
BMD _{Callus} (mg HA/cc)	774 (28)	667 (16)	p<0.005
BMC _{Callus} (mg HA)	1.62 (0.47)	2.7 (0.76)	p<0.005
Union Ratio	0.129 (0.088)	0.277 (0.068)	p<0.005

Table 3

Micro CT-derived Parameters of the unoperated contralateral femurs at 6 weeks, presented as mean (standard deviation). P values indicate significant differences between the teriparatide treated samples and the saline treated controls, while NS indicates no significant differences.

	Saline	Teriparatide	
Medial-Lateral Periosteal Diameter (mm)	1.76 (0.03)	1.85 (0.08)	NS
Anterior-Posterior Periosteal Diameter (mm)	1.22 (0.001)	1.30 (0.05)	NS
Cortical Thickness (mm)	0.180 (0.006)	0.199 (0.007)	p<0.05
Cortical Bone Density (mg HA/cc)	1201 (4)	1179 (21)	NS
Cross-Sectional Area (mm ²)	0.69 (0.01)	0.82 (0.05)	p<0.05
Polar Moment of Inertia (mm ⁴)	0.27 (0.01)	0.36 (0.03)	p<0.05

Table 4

Coefficients of determination (R^2) from univariate linear regression of micro CT parameters with torsional biomechanical properties. Significant correlations are in bold face. Asterisk indicates the best univariate predictor for each of the biomechanical properties.

	Torsional Rigidity		Yield Torque		Ultimate Torque	
	R^2	<i>p-value</i>	R^2	<i>p-value</i>	R^2	<i>p-value</i>
PMI _{Min}	0.38	0.001	0.42	0.001	0.57*	0.001
PMI _{Ave}	0.09	0.124	0.02	0.452	0.10	0.114
PMI _{Max}	0.02	0.448	0.14	0.051	0.02	0.477
BV _{Graft}	0.01	0.622	0.04	0.371	0.05	0.356
BV _{Callus}	0.29	0.004	0.14	0.055	0.20	0.018
BV _{Intramed}	0.31	0.002	0.19	0.022	0.16	0.038
BMD _{Callus}	0.43	0.001	0.18	0.028	0.07	0.176
BMC _{Callus}	0.20	0.019	0.09	0.130	0.16	0.037
Union Ratio	0.77*	0.001	0.59*	0.001	0.30	0.003