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Engraftment and bone mass are enhanced by PTHrP 1-34 in ectopically transplanted vertebrae (vossicle model) and can be non-invasively monitored with bioluminescence and fluorescence imaging

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Abstract

Evidence exists that parathyroid hormone-related protein (PTHrP) 1-34 may be more anabolic in bone than parathyroid hormone (PTH) 1-34. While optical imaging is growing in popularity, scant information exists on the relationships between traditional bone imaging and histology and bioluminescence (BLI) and fluorescence (FLI) imaging. We aimed to evaluate the effects of PTHrP 1-34 on bone mass and determine if relationships existed between radiographic and histologic findings in bone and BLI and FLI indices. Vertebrae (vossicles) from mice coexpressing luciferase and green fluorescent protein were implanted subcutaneously into allogenic nude mice. Transplant recipients were treated daily with saline or PTHrP 1-34 for 4 weeks. BLI, FLI, radiography, histology, and μ CT of the vossicles were performed over time. PTHrP 1-34 increased bioluminescence the most after 2 weeks, fluorescence at all time points, and decreased the time to peak bioluminescence at 4 weeks ($P = 0.027$), the latter of which suggesting enhanced engraftment. PTHrP 1-34 maximized vertebral body volume at 4 weeks ($P < 0.0001$). The total amount of bone observed histologically increased in both groups at 2 and 4 weeks ($P = 0.002$); however, PTHrP 1-34 exceeded time-matched controls ($P = 0.044$). A positive linear relationship existed between the percentage of trabecular bone and **1**) total bioluminescence ($r = 0.595$; $P = 0.019$); **2**) total fluorescence ($r = 0.474$; $P = 0.074$); and **3**) max fluorescence ($r = 0.587$;

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COMPLIANCE WITH ETHICAL STANDARDS

Statement on the welfare of animals: All applicable institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted, with all experimentation being approved by the Institutional Animal Care and Use Committee (IACUC 2009A0162). This article does not contain any studies with human participants performed by any of the authors.

$P = 0.021$). In conclusion, PTHrP 1-34 enhances engraftment and bone mass, which can be monitored non-invasively by BLI and FLI.

Keywords

PTHrP; vossicle; transplantation; bioluminescence; fluorescence

INTRODUCTION

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and the microarchitectural deterioration of bone. Fracture prevention is the primary goal of treatment, of which bone resorption-inhibiting bisphosphonates are currently the mainstay of therapy (Grey 2007). However, based on side-effect concerns and the limited utility of bisphosphonates in the face of advanced disease, there is a need to develop drugs that will increase bone formation, rather than prevent resorption (Lewiecki 2003). In addition, up to 20% of traditional fracture repairs result in delayed or non-union, and bone grafts used in skeletal repair often experience delayed or failed engraftment (Brigman et al. 2004; Marsh 1998). Since bone formation and angiogenesis are critically linked processes, the development of agents that could potentially enhance engraftment, as well as bone formation, would be advantageous in orthopedic applications.

Parathyroid hormone (PTH) was the first skeletal anabolic agent approved for treating osteoporosis (Reeve 2002). The increase in BMD, and ultimately bone strength, linked to its use is associated with a reduction in overall fracture risk in vertebrae between 65 and 71% and in non-vertebral sites of 35 to 68% (Lindsay et al. 2004). The bone-forming effects of PTH have since been extended to, and investigated in, other orthopedic applications, including routine fracture repair, fracture non-union and delayed union, implant osseointegration, distraction osteogenesis, spinal fusion, and allograft incorporation (Dhillon et al. 2013; Ming et al. 2012; Oteo-Alvaro and Moreno 2010; Seebach et al. 2004; Skripitz and Aspenberg 2001). Recently, PTH has been demonstrated to have pro-angiogenic effects in bone (Dhillon et al. 2013).

The search for the ideal bone-forming agent has led to the investigation of parathyroid hormone-related protein (PTHrP), since PTHrP is structurally and functionally similar to PTH within their N-terminal regions (PTHrP 1-34 and PTH 1-34) (Civitelli et al. 1989). However, evidence suggests that PTHrP 1-34 may be a more potent anabolic compound in bone than PTH 1-34. This has been demonstrated by the fact that while both peptides increase bone mass, treatment with PTHrP 1-34 results in **1)** a peripheral increase in bone formation markers with no change in bone resorption markers; **2)** histologic evidence of bone formation in the absence of bone resorption; and **3)** an absence of hypercalcemia (Horwitz et al. 2003; Riccardi 2012). These findings suggest that PTHrP 1-34 may be more efficacious for the treatment of osteoporosis and in other orthopedic applications (Bisello et al. 2004).

Optical imaging is gaining popularity to study biologic processes in a rapid, cost-effective, and non-invasive manner when compared to traditional imaging modalities (Choy et al.

2003). This is very valuable in longitudinal studies due to both a reduction in animal numbers and total radiation dose. The most common uses of bioluminescence (BLI) and fluorescence (FLI) imaging includes the study of cancer progression and monitoring the behavior of transplanted cells and tissues (Andrade et al. 2011; Choy et al. 2003; Martin et al. 2010). While the utility of optical imaging in musculoskeletal drug discovery and regenerative medicine studies is increasing, scant information exists on the relationship between traditional imaging and the true histologic assessment of bone and optical imaging findings (Ben Arav et al. 2012; Degano et al. 2008; Sheyn et al. 2013).

Vossicles are engrafted thoracolumbar vertebrae into the subcutis of mice and represent a dynamic model of osteogenesis (Liao et al. 2008; Pettway and McCauley 2008). Vossicles have clear advantages over implanted cells or cell-based scaffolds, including **1)** an established bone microarchitecture and microenvironment and **2)** the capability to be used to evaluate the response of bone as a tissue to skeletal therapeutics, genetic modifications, and bone-cancer interactions.

To study the effects of PTHrP 1-34 on bone mass *in vivo* in both a pre-established bone microenvironment and using BLI and FLI, we employed vossicles from mice coexpressing the bioluminescent protein luciferase (Luc) and green fluorescent protein (GFP). The purpose of this study was to determine **1)** the effects of PTHrP 1-34 on bone mass *in vivo*; **2)** if PTHrP 1-34 would enhance vossicle engraftment; and **3)** if relationships existed between histologic and radiographic indices of bone mass and BLI and FLI indices. We hypothesized that **1)** PTHrP 1-34 would enhance vossicle engraftment and bone mass and **2)** linear relationships would exist between histologic, radiographic, BLI, and FLI indices.

MATERIALS AND METHODS

Animals

Vertebral allografts (vossicles) were isolated from 5-day-old FVB-Tg(CAG-luc-GFP)L2G85Chco/J mice (The Jackson Laboratory, Bar Harbor, ME). These mice express the CAG-luc-GFP L2G85 transgene and therefore express Luc and GFP under the direction of the human cytomegalovirus immediate early promoter enhancer with a chicken beta-actin/rabbit beta-globin hybrid promoter (CAG). These mice will be referred to as Luc-GFP mice. Bioluminescence resulting from constitutive Luc activity is detected in all cells except mature erythrocytes. No GFP activity is detected in any hematopoietic tissue, as determined by **1)** flow cytometric analysis reported by The Jackson Laboratory and **2)** a lack of fluorescence in the vasculature of these mice (our unpublished results). Allograft donor parents were confirmed to be homozygous for the transgene by both **1)** RT-PCR using genomic DNA and primers specific for the GFP allele (5' – AAG TTC ATC TGC ACC ACC G – 3' [Forward] and 5' – TCC TTG AAG AAG ATG GTG CG – 3' [Reverse]) and **2)** positive confirmation of bioluminescence (IVIS 100 System, PerkinElmer, Santa Clara, CA). Vossicle donor pups were produced by breeding of these mice and were confirmed to be positive for bioluminescence prior to euthanasia. Transplant recipients were allogenic 6–8 week old male homozygous CrTac:NCr-Foxn1tm athymic nude mice (Taconic Farms, Inc., Hudson, NY). All applicable institutional guidelines for the care and use of animals were

followed and all experimentation was approved by the Institutional Animal Care and Use Committee (IACUC 2009A0162).

Vossicle implantation

Surgical instruments were initially steam sterilized and cleaned and re-sterilized with 100% ethanol and a hot bead sterilizer between surgical patients. Donor pups were euthanized with CO₂ asphyxiation and cervical dislocation. Pups were positioned in sternal recumbency and the dorsal skin overlying the spine sanitized with 100% ethanol. One-centimeter long craniocaudal skin incisions were made over their thoracolumbar vertebral region with a #15 scalpel blade. Thoracolumbar vertebral chains (Liao et al. 2008; Pettway and McCauley 2008) were excised by sharply transecting the vertebral column at the level of T12/T13 and L6/S1. The musculature and associated soft tissues were dissected from the lateral and ventral aspects of the vertebrae. Vossicles were rinsed in sterile saline (1X pH 7.4 phosphate-buffered saline, Life Technologies, Grand Island, NY). Fifteen male athymic nude mice (7 – 8 per treatment group), serving as transplant recipients, were anesthetized individually in an induction chamber with 5% isoflurane (IsoFlo®, Abbott Animal Health, Abbott Park, IL) in 3 L/min oxygen and maintained with 2% isoflurane in 2 L/min oxygen delivered with a face mask. Eyes were protected using ophthalmic lubricant and mice were positioned on a warm water blanket in sternal recumbency. The dorsum was surgically prepared with 100% ethanol followed by five minutes of contact time with chlorhexidine. A total of 4 craniocaudal 5–8 mm skin incisions were made; 2 caudal to the shoulder blades in the upper left and right quadrants of the dorsum and 2 cranial to and overlying the pelvis in the lower left and right quadrants of the dorsum. The subcutaneous fat was undermined on the lateral aspects of each skin incision and 1 vertebral chain placed in this space. Incisions were closed with a subcutaneous buried simple interrupted pattern using 5-0 polydioxanone (PDS-II, Ethicon Endo-Surgery, Inc., Somerville, NJ) and the skin edges apposed with n-butyl cyanoacrylate (Vetbond™ Tissue Adhesive, 3M™, St. Paul, MN).

Treatment with PTHrP 1-34

Recipient nude mice were assigned to either one of two treatment groups: **1)** saline control or **2)** 80 µg/kg of PTHrP 1-34 (Bachem Americas, Inc., Torrance, CA) dissolved in sterile saline (Opas et al. 2000; Xu et al. 2013). The amino acid sequence of PTHrP 1-34 is identical between man, rat, and mouse. Mice were briefly anesthetized as described above and treated every day beginning one day after vossicle implantation with a subcutaneous injection of either saline or PTHrP 1-34 on the dorsum cranial to the shoulder blades and distant from the vossicles. Brief anesthesia was utilized to facilitate injection and to minimize the potential of traumatizing the implants that could occur with manual restraint of a conscious mouse. Mice were weighed at the time of vossicle implantation and once weekly during the 4-week treatment period to calculate an exact drug dosage and ensure mice did not meet early removal criteria (10% loss of body weight).

Bioluminescence imaging (BLI)

Nude mice bearing vossicles were imaged at the time of implantation and at 2 and 4 weeks after implantation. Mice were anesthetized as described above and injected intraperitoneally

with 4.3 mg of D-luciferin in a volume of 100 μ l sterile saline. Fifteen to thirty second acquisitions of *in vivo* bioluminescence began immediately and then every 30 seconds thereafter (PerkinElmer). Mice were imaged until the peak bioluminescence signal was obtained. Analyses were performed using the same pre-determined color threshold encompassing the entire range of individual photon signals emitted from all vossicles at all time points. Tracings of each vossicle's bioluminescence were performed using the software's automatic tracing feature and visually checked for accuracy. Peak total bioluminescence flux (photons/second) and the time to peak bioluminescence (min) were calculated.

Fluorescence imaging (FLI)

Prior to imaging the vossicles *in situ*, a custom filter setting was established (described below) to separate and quantify the amount of GFP in luc-GFP vossicles from background autofluorescence of the recipient mice using the Maestro EX 2.10 System (Cambridge Research & Instrumentation, Inc. [CRI], Woburn, MA). Age-matched 5-day-old luc-GFP and naïve C57BL/6 mice (age of implantation) and 4-week-old luc-GFP and naïve C57BL/6 mice (age of longest implantation) were used in combination to determine the optimal filter setting over time. In brief, the amount of GFP signal was determined by subtracting the fluorescent spectra from the lumbar spine of C57BL/6 mice from the fluorescent spectra of the lumbar spine of age-matched luc-GFP mice. This resulted in a single peak corresponding to the GFP signal emitted from luc-GFP mice. Recipient mice were anesthetized as previously described and maintained individually within the Maestro imaging cabinet. Using our custom filter setting, total and max fluorescence counts were measured at 2 and 4 weeks from *in situ* vossicles. Quantification of fluorescence at the time of implantation was not performed due to the fact the cyanoacrylate tissue glue used to close the skin incisions interfered with the detection of the vossicles.

Vossicle explantation

Mice were anesthetized after both BLI and FLI and prepared for surgery as described for implantation. A single vossicle was removed at 2 and 4 weeks for analysis by weighing, digital radiography, microcomputed tomography (μ CT), and histomorphometry. The surgical wounds were closed as described. Vossicles were fixed in formalin for 48 hours and then maintained in 70% ethanol at 4° C. Vossicles were weighed just prior to digital radiography.

Digital radiography

Digital high-resolution orthogonal ventrodorsal (VD) and lateral radiographic images were taken of each explanted vossicle using the LX-60 Laboratory Radiography System (Faxitron Bioptics, LLC, Tucson, Arizona). The system was calibrated before use and all vossicles were radiographed during the same imaging session. The number of vertebral bodies within the implant were counted. The overall cross-sectional areas of the most cranial and caudal complete individual vertebral bodies of the vertebral chains were measured on VD radiographic views using imaging software (OsiriX, Geneva, Switzerland). Vertebral body heights were measured on the same vertebrae on the lateral radiographic views. Individual

vertebral body volumes (VBVs) were then calculated by multiplying the vertebral cross-sectional areas by their corresponding vertebral body heights. Individual VBVs were averaged between the two end vertebrae for each vossicle for comparison between groups. A strong, statistically significant positive correlation exists between VBV determined radiographically by this method and VBV determined by μ CT (Pearson $r = 0.955$, $P < 0.0001$, $n=10$, unpublished observations). Therefore, this digital radiographic determination of VBV is acceptable to determine the volume of individual vertebral bodies.

Microcomputed tomography

The vossicle with the peak total bioluminescence flux measurement closest to the mean for their respective group ($n = 1$) was chosen for imaging by μ CT (Inveon Preclinical CT scanner, Siemens Medical Solutions USA, Inc., Malvern, PA). This was performed to both visually represent the implant and to complement the radiographic and histologic findings. Images were acquired in ~441 projections over 360 degrees at 110 kVp, 150 MA, 1300 millisecond exposure, Bin 1, and a medium-high system magnification with an isotropic pixel width (resolution) of 9.7 μ m. Image data was reconstructed using Cobra software (Exxim, Pleasanton, CA). Bone morphometric indices – trabecular volume/total volume (Tb.V/TV, %), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, μ m), trabecular separation (Tb.Sp, μ m), and trabecular pattern factor (Tb.Pf) – were analyzed in the caudal-most vertebral body using 3D bone morphometry analysis software (Inveon Research Workplace 3D Image Software, Siemens PreClinical). Segmentation thresholds were kept constant for all treatment groups. Two-dimensional VD views were created (RATOC System Engineering Co, Tokyo, Japan).

Histomorphometry

After advanced imaging, 3–5 randomly selected vossicles per group were moved from 70% ethanol into 10% EDTA solution, pH = 7.4. Vossicles were decalcified on a shaker at 4° C for 2 weeks, changing the 10% EDTA once every 3 days. Vossicles were hand embedded on their lateral edge in paraffin. Sectioning was performed at 5 μ m thickness until midline sections were obtained. Slides were then stained with hematoxylin and eosin. Vossicles were imaged at 100X magnification by light microscopy with a digital camera system (NIS Elements F 4.00.00, Nikon Instruments Inc., Melville, NY). Histologic interpretation and histomorphometric analyses were performed in a random order. The overall cross-sectional areas of the most cranial and caudal complete vertebral bodies of the vertebral chains were measured in the sagittal plane and the percentage of the vertebrae that was **1**) bone; **2**) cortical bone; and **3**) trabecular bone were calculated (Aperio Imagescope v 11.2.0.780, Leica Microsystems Inc., Buffalo Grove, IL). These percentages were averaged between the two end vertebrae for each vossicle for comparison between groups.

Statistical analysis

Values are represented as mean $\pm$ 1 standard deviation. Due to the fact that a chain of thoracolumbar vertebrae were implanted, vossicle weight, total bioluminescence flux, and total and max fluorescence counts were normalized to the exact number of vertebral bodies implanted (determined radiographically after explantation) for comparison between groups.

All data was normally distributed and had equal variance except total bioluminescence flux, total fluorescence counts, and max fluorescence counts, which achieved normality and equal variance following base-10 logarithmic transformation of the data. Therefore, parametric statistical analyses were used for all comparisons. A one-way ANOVA and a Newman-Keuls post-hoc analysis was used to compare the following variables over time after treatment with either saline or PTHrP 1-34: total bioluminescence flux, vossicle weight, the cubed root of individual VBV, and the percentage of total bone, cortical bone, and trabecular bone within the vertebrae. A one-way ANOVA and a Dunnett's multiple comparisons test was used to compare the time of peak bioluminescence of each treatment group at 2 and 4 weeks to that of the time of implantation. Unpaired t-tests were used to compare the time of peak bioluminescence and both total and max fluorescence counts between saline and PTHrP 1-34-treated vossicles at 2 and 4 weeks. A Pearson correlation matrix analysis was performed using control and PTHrP 1-34-treated vossicles from the 2 and 4 week time points to determine if linear relationships existed between variables included in this study. Analyses were performed with commercial software (GraphPad Prism v6.00, GraphPad Software, Inc., San Diego, CA and SigmaStat v3.5, Systat Software, Inc., Chicago, IL) with statistical significance established at $P < 0.05$.

RESULTS

PTHrP 1-34 significantly increased fluorescence, but not bioluminescence, when compared to time-matched controls

No mice exhibited adverse effects due to vossicle implantation or treatment with PTHrP 1-34. Only 4 vossicles were analyzed at 2 weeks in the control group due to implanting one less vossicle in three of these mice. Treatment with saline and PTHrP 1-34 for 2 weeks resulted in a significant increase in total bioluminescence when compared to the time of implantation ($P = 0.026$, Figure 1b). Despite the relative increase in total bioluminescence at 4 weeks following treatment with PTHrP 1-34 when compared to baseline, this did not reach statistical significance ($P = 0.127$). In addition, the increase in total bioluminescence following treatment with PTHrP 1-34 at 2 and 4 weeks when compared to time-matched controls did not reach statistical significance (Figure 1b). There was a significant increase in both total and max fluorescence counts after treatment with PTHrP 1-34 at both 2 and 4 weeks when compared to time-matched controls ($P = 0.027$, Figures 1d and 1e).

PTHrP 1-34 decreased the time to peak bioluminescence, suggesting enhanced vossicle engraftment

Vossicles in both treatment groups at 2 and 4 weeks exhibited a significant decrease in the time to peak bioluminescence when compared to the time of implantation ($P < 0.0001$, with an adjusted threshold for significance of $P = 0.025$ due to multiple comparisons, Figure 1c). Treatment with PTHrP 1-34 resulted in a more rapid time to peak bioluminescence at 4 weeks when compared to time-matched controls ($P = 0.004$, with an adjusted threshold for significance of $P = 0.025$ due to multiple comparisons, Figure 1c).

Vossicle mass and individual VBV increased over time, with PTHrP 1-34 enhancing VBV

Control and PTHrP 1-34-treated mice had a significant increase in vossicle weight at 4 weeks when compared to baseline ($P = 0.006$, Figure 2). However, no differences existed between treatment groups at either 2 or 4 weeks. There was a significant increase in individual VBVs within the vertebral chain after 2 weeks in both treatment groups ($P = 0.003$), which further increased after 4 weeks of implantation ($P = 0.002$, Figures 3a and 3b). Treatment with PTHrP 1-34 for 4 weeks resulted in the greatest increase in VBV, exceeding all other treatment groups with significance ($P < 0.0001$, Figures 3a and 3b). The radiographic assessment of individual VBVs within the vertebral chain (Figure 3a) were supported by observing μ CT scans of representative vossicles for each group (Figure 3c).

The amount of bone present within the vertebrae peaked at 2 weeks, but was enhanced at both 2 and 4 weeks in vossicles treated with PTHrP 1-34

Upon histologic analysis, the vertebral bodies present within the vossicles were surrounded by a thin layer of skeletal muscle at the time of implantation. The vertebral arches were completely cartilaginous and the trabecular structures within all of the vertebral bodies contained both cartilage and more mature mineralized bone. After 2 and 4 weeks of implantation, there was 100% viability of all dorsal vertebral arches, as indicated by progressive maturity from completely cartilaginous structures to mature bone (data not shown). However, only the most cranial and caudal end 1–2 vertebral bodies within the vertebral chain were viable with maintenance of bone architecture. Conversely, the more centrally-located vertebral bodies within the vertebral chain were avascular and lytic at 2 weeks. This lysis progressed over 4 weeks in both treatment groups. The skeletal muscle surrounding the vertebral bodies was progressively replaced by fibrous and adipose tissue over 4 weeks of implantation.

Representative images of end vertebral bodies from the vossicles at the time of implantation and after 2 and 4 weeks of treatment with either saline or PTHrP 1-34 are presented (Figure 4a). Quantitatively, the overall total percentage of bone in the end vertebrae was significantly greater in saline and PTHrP 1-34-treated vossicles at 2 and 4 weeks when compared to the time of implantation ($P = 0.002$, Figure 4b). Treatment with PTHrP 1-34 for 2 and 4 weeks resulted in a greater total amount of bone within the vertebrae than time-matched controls ($P = 0.044$, Figure 4b). Closer evaluation of the cortical and trabecular bone compartments revealed a significant increase in the percentage of cortical bone within the vertebrae in both treatment groups at 2 ($P = 0.012$) and 4 weeks ($P < 0.001$) when compared to the time of implantation (Figure 4c). However, there was no difference in the amount of cortical bone between treatment groups at 2 and 4 weeks. Evaluation of the trabecular bone compartment revealed that the increase in the overall amount of bone within the vertebrae in the PTHrP 1-34 treatment group at both 2 and 4 weeks when compared to the time of implantation and time-matched controls was due to an increase in the amount of trabecular bone ($P = 0.014$, Figure 4d). Despite these findings, the percentage of total bone within the vertebrae decreased at 4 weeks after peaking at 2 weeks (Figure 4b). This resulted from a significant decrease in trabecular bone area in both treatment groups ($P = 0.020$, Figure 4d). These changes observed within the trabecular bone compartment histologically were also observed upon μ CT analysis of one representative vossicle per group (Figure 3d).

Strong agreement existed between trabecular bone area (2D assessment by histology) and trabecular bone volume (Tb.V/TV, 3D evaluation by μ CT) (Pearson $r = 0.895$, $P = 0.04$, $n=5$). The Tb.V/TV was greater in PTHrP 1-34-treated vossicles at both 2 and 4 weeks, however decreased in both treatment groups from 2 to 4 weeks (Figure 3d). This increase in Tb.V/TV in PTHrP 1-34-treated vossicles was associated with an **1**) increase in Tb.N and a **2**) decrease in the Tb.Pf, when compared to control vossicles at 2 and 4 weeks (Figure 3d).

Association between radiographic, histologic, and functional (BLI and FLI) indices

A significant positive correlation existed between individual VBVs within the vertebral chain and vertebral weight ($r = 0.689$; $P = 0.0001$) (Figure 5). Histologically, a significant positive linear relationship was present between the overall total percentage of bone within the vertebrae and the percentage of trabecular bone ($r = 0.924$; $P < 0.0001$) (Figure 5). A significant negative correlation was observed between the percentages of trabecular and cortical bone ($r = -0.578$; $P = 0.019$). Interestingly, a positive linear relationship that approached statistical significance existed between the percentage of total bone observed within the vertebrae histologically and **1**) total bioluminescence ($r = 0.503$; $P = 0.056$); **2**) total fluorescence ($r = 0.494$; $P = 0.061$); and **3**) max fluorescence ($r = 0.466$; $P = 0.080$) (Figure 5). Closer examination revealed a positive linear relationship between the percentage of trabecular bone and **1**) total bioluminescence ($r = 0.595$; $P = 0.019$); **2**) total fluorescence ($r = 0.474$; $P = 0.074$); and **3**) max fluorescence ($r = 0.587$; $P = 0.021$) (Figure 5). In addition, a significant negative correlation existed between individual VBVs within the vertebral chain and total bioluminescence ($r = -0.431$; $P = 0.040$) (Figure 5). Interestingly, a negative but non-significant relationship existed between the percentage of cortical bone and **1**) total bioluminescence ($r = -0.444$; $P = 0.097$); **2**) total fluorescence ($r = -0.163$; $P = 0.561$); and **3**) max fluorescence ($r = -0.498$; $P = 0.059$) (Figure 5). As expected, a significant positive correlation was observed between total bioluminescence and both **1**) total fluorescence ($r = 0.412$; $P = 0.045$) and **2**) max fluorescence ($r = 0.509$; $P = 0.011$).

DISCUSSION

The goals of this study were to determine **1**) the effects of PTHrP 1-34 on bone mass *in vivo*; **2**) if PTHrP 1-34 would enhance vossicle engraftment; and **3**) if relationships existed between histologic and radiographic indices of bone mass and BLI and FLI indices.

Treatment with PTHrP 1-34 for 4 weeks increased VBV, indicating its utility in increasing bone mass. This finding parallels clinical evidence that PTH 1-34 increases lumbar vertebral size (Schnell et al. 2010). From a time-course perspective, PTH 1-34 enhanced spinal fusion and implant osseointegration after 4 weeks (Ming et al. 2012; Skripitz and Aspenberg 2001). In addition, we treated mice with 80 μ g/kg/day, of which the same dose of PTH 1-34 increases bone formation (Opas et al. 2000; Xu et al. 2013). These results are consistent with our findings. We implanted vossicles from 5-day-old mice. The robust increase in radiographic and histologic indices of bone mass in both treatment groups may result from the fact that modeling-based bone formation accelerates in the neonatal period (Kovacs 2011). With specific regard to the increase in bone mass observed in this study after

treatment with PTHrP 1-34, the osteogenic effects of PTH 1-34 are the most pronounced in rapidly growing animals. This results from the fact that PTH 1-34 stimulates mainly modeling-based bone formation (Bellido et al. 2013).

Histologic analyses revealed that the percentage of total bone within the vossicles peaked at 2 weeks in both treatment groups, with treatment with PTHrP 1-34 resulting in a more robust and statistically significant increase in total histologic bone mass at both 2 and 4 weeks. This response resulted from, and was parallel to, changes in trabecular bone, despite an increase in the amount of cortical bone over time. These trabecular changes were supported by our μ CT analysis. The Tb.V/TV was greater in PTHrP 1-34-treated vossicles at both 2 and 4 weeks, however decreased in both treatment groups by 4 weeks. This increase in PTHrP 1-34-treated vossicles was associated with **1)** an increased Tb.N and **2)** a decreased Tb.Pf when compared to controls. A decrease in Tb.Pf indicates better trabecular connectivity. Therefore, PTHrP 1-34 improved both trabecular number and connectivity, similar to PTH 1-34 (Jerome et al. 2001).

The percentage of both total and trabecular bone decreased from 2 to 4 weeks in both treatment groups, resulting from VBVs increasing more rapidly than absolute measurements of total and trabecular bone mass. This explains the discrepancy between increased VBVs/vossicle weights and the decreased percentages of total and trabecular bone from 2 to 4 weeks. Since we implanted vertebrae from 5-day-old mice, the implants are ~3 to 5 weeks of age after 2 and 4 weeks. There is a normal decrease in the trabecular bone percentage in mice from ~1 to 2 months of age (Moverare-Skrtic et al. 2014; Thongchote et al. 2014). Therefore, the decrease in the percentage of total and trabecular bone between 2 and 4 weeks partially results from normal age-related changes. In addition, vossicles are relatively “unloaded” since they are not in the same mechanical environment as the spine *in situ*. Since trabecular bone is more metabolically active and responsive to changes in mechanical loading, part of the decline in trabecular and total bone from 2 to 4 weeks may result from the vossicles being in an unloaded environment.

PTHrP 1-34 decreased the time to peak bioluminescence. A more rapid time to peak bioluminescence has been shown to indicate enhanced vascularization (Inoue et al. 2010). Since achieving rapid vascularization is key to successful engraftment in any area of transplantation, this finding with PTHrP 1-34 is of paramount importance. While we did not histologically assess engraftment or angiogenesis, the significant reduction in the time to peak bioluminescence with PTHrP 1-34 is consistent with, and highly suggestive of, enhanced engraftment. Angiogenesis and bone formation are critically linked biological processes. Pro-angiogenic and pro-osteogenic factors are made by osteoblasts or vascular endothelial cells in response to PTH 1-34 or PTHrP 1-34. In addition, PTH 1-34 increases **1)** vascularity in irradiation bone and **2)** bone formation in and osseointegration of devitalized allografts into bone defects (Kang et al. 2013; Reynolds et al. 2011; Sheyn et al. 2013). Therefore, our findings suggest that PTHrP 1-34-enhanced engraftment may contribute to enhanced osteogenesis. Clinical patients and mouse models of osteoporosis have reduced bone vascularity and perfusion, suggesting a vascular component to the disease (Griffith et al. 2005; Shih et al. 2004; Zhao et al. 2012). Since drugs that enhance vascularization are lacking, PTHrP 1-34 may represent a novel therapeutic approach in bone regeneration.

We used BLI and FLI in an attempt to non-invasively monitor bone mass. Studies monitoring the biological response of transplanted tissues other than bone have shown that BLI and FLI indices have linear relationships with other imaging modalities and outcome variables (Abou-Elkacem et al. 2011; Choy et al. 2003). Mice expressing luciferase under the control of the type I collagen promoter have been used to study the effect of PTH 1-34 in bone (Opas et al. 2000). Therefore, luciferase was expressed in type I collagen-producing cells, including osteoblasts. Similar to our study, this work demonstrated that 80 µg/kg/day of PTH 1-34 increased **1)** luciferase activity 2–3 fold after 11 days (~2 weeks) and **2)** both luciferase activity (< 1-fold) and bone formation after 4 weeks when compared to controls. However, this study differed from ours. While luciferase expression was almost bone-exclusive in their study, *in vivo* BLI or FLI was not performed, and luciferase activity was measured in post-mortem tibial extracts. Histomorphometry was performed on dissected femurs only after 4 weeks of treatment. Lastly, while luciferase activity paralleled the rate of bone formation, correlation analysis was not performed (Opas et al. 2000). In our current study, we found a positive linear relationship between both the percentage of total and trabecular bone observed histologically and **1)** total bioluminescence flux; **2)** total fluorescent counts; and **3)** max fluorescence counts that either reached, or approached, statistical significance. Since we only evaluated 3–5 vossicles per treatment group histologically, the lack of a significant correlation between the percentage of total bone and total bioluminescence ($P = 0.056$), total fluorescence ($P = 0.061$), and max fluorescence ($P = 0.080$) is partially due to the small sample size. However, we observed a higher correlation coefficient and lower P value with BLI when assessing the correlation between both total and trabecular bone and optical imaging findings. This indicates that BLI is a better predictor of the percentage of total and trabecular bone than FLI using GFP. An alternative approach would be to employ fluorescence proteins in the red and near-infrared range, to maximize tissue penetration and reduce tissue absorption and autofluorescence from non-target tissues (Shcherbo et al. 2007; Shcherbo et al. 2010). In addition, using tissue-specific regulatory elements, such as promoters specific to bone-forming osteoblasts, could also be considered. These potential modifications would have likely further strengthened the linear relationships between histologic and FLI indices. Despite this, total and max fluorescence were significantly greater at 2 and 4 weeks after treatment with PTHrP 1-34. The lack of a significant increase in total bioluminescence flux following therapy with PTHrP 1-34 when compared to time-matched controls is likely due to the limitations of BLI: **1)** potential differences in D-luciferin pharmacokinetics between mice and **2)** high signal variability. The latter results from BLI requiring both an exogenous substrate (D-luciferin) and endogenous luciferase activity and not just protein expression as with FLI. Despite this, linear relationships did in fact exist between histologic bone mass and BLI and FLI indices. Therefore, in this model, BLI and FLI, alone or in combination, can be used to evaluate highly relevant processes in osteogenesis – engraftment and treatment response - while longitudinally and non-invasively assessing graft activity. This assessment of bone mass can likely be extended to other orthopedic applications.

An alternative would have been to perform *in vivo* µCT, which can be used to non-invasively and longitudinally measure bone development, response to therapy, and disease. An advantage of *in vivo* µCT is a reduction in animal numbers by evaluating the same

anatomical site over time (Bouxsein et al. 2010). The main disadvantage in longitudinal studies is the cumulative radiation dose (of which young, growing mice are uniquely sensitive), which can significantly reduce bone mass. In addition, motion artifacts from breathing are an issue, particularly at higher resolutions (Bouxsein et al. 2010). Lastly, equipment and operational costs can be prohibitive. While we could have performed *in vivo* μ CT, vossicle removal allowed for histologic assessment and correlation of these findings with concurrent BLI, FLI, and radiographic indices. In addition, the limitations of *in vivo* μ CT, particularly since our vossicles were **1)** derived from 5-day old mice (cumulative radiation dose) and **2)** implanted on the dorsum of nude mice (motion artifacts), may be detrimental in this vossicle model.

While it is feasible to employ solely naïve Luc-GFP mice to assess the effects of PTHrP 1-34 on bone mass rather than transplanting their vertebrae, our vossicle model has distinct advantages. First, vossicles have benefits over implanted cells, cell-based scaffolds, and *in situ* skeletal evaluation, including: **1)** an established bone microarchitecture and microenvironment and **2)** the ability to study bone in genetically-modified mice that may have debilitating or perinatal lethal mutations. Vossicles expressing Luc-GFP allow for the non-invasive measurement of **1)** engraftment and **2)** changes in bone mass over time. There would be several limitations of using BLI and FLI to assess the effects of PTHrP 1-34 on the bones of naïve Luc-GFP mice *in situ*. First, hair scatters and attenuates bioluminescence (Badr 2014) and decreases fluorescence. Second, background fluorescence arises from food or bedding debris. While removing the hair of naïve Luc-GFP mice would overcome these limitations, resultant skin irritation and alterations in pigmentation attenuates bioluminescence and fluorescence from deeper tissues (Badr 2014; Curtis et al. 2011). Therefore, it is suggested that mice lacking hair, such as nude mice, be used for optical imaging (Badr 2014). Lastly, isolating the BLI or FLI signal emitted from one specific anatomical site, such as the lumbar spine, in naïve Luc-GFP mice would be challenging due to **1)** the inability of BLI and FLI to delineate distinct boundaries of a bone and **2)** signal arising from surrounding tissues.

A limitation of implanting a chain of vertebrae was their engraftment pattern. At the time of explantation, the entire vossicle was attached solely to the host's subcutaneous tissue, with no evidence of attachment to the underlying skeletal muscle. This is consistent with other reports of subcutaneous bone implantation (Felts 1961). Histology revealed 100% viability of all of the dorsal vertebral arches, but only the end 1–2 vertebral bodies were viable with maintenance of bone architecture. This coincides with **1)** these regions being attached dorsally and at the ends to the subcutis and **2)** vertebral bodies being separated by intervertebral discs. For this reason, we analyzed only the end vertebrae radiographically and histologically, averaging their measurements for statistical analysis. This also led to equal representation of thoracolumbar (cranially) and lumbar (caudally) vertebrae. This indicates that in the future, only vossicles consisting of 2–3 vertebral bodies should be used or if wishing to implant a chain of vertebrae, they should be implanted upside down. This would facilitate 100% engraftment of the vertebral bodies into the subcutis, like the vertebral arches and end vertebral bodies did in this study.

Bone development and formation studies have been limited by the lack of non-invasive, quantitative techniques. This vossicle model may aid in therapy development by allowing the non-invasive assessment of bone. Our findings show that PTHrP 1-34 enhances engraftment and bone mass, supporting the use of BLI and FLI to non-invasively monitor bone mass.

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Fig. 1. Representative vossicle BLI images (a), total bioluminescence flux (photons/second) (b), and time of peak bioluminescence (min) (c) at the time of implantation and after 2 and 4 weeks of treatment with either saline or PTHrP 1-34 and total fluorescence (counts) (d) and max fluorescence (counts) (e) after 2 and 4 weeks of treatment with either saline or PTHrP 1-34. For all figures, “A” indicates a significant difference from the time of implantation and “B” indicates a significant difference from time-matched controls. Subfigure (a) represents *in situ* images of the peak total bioluminescence flux signal obtained from a representative vossicle for each treatment group. The BLI images were obtained and analyses were performed using the same pre-determined color threshold encompassing the entire range of individual photon signals emitted from all vossicles at all time points. Treatment with saline and PTHrP 1-34 for 2 weeks resulted in a significant increase in total bioluminescence when compared to the time of implantation. Despite the relative increase in total bioluminescence at 4 weeks following treatment with PTHrP 1-34 when compared to baseline, this did not reach statistical significance ($P = 0.127$). Both treatment groups at 2 and 4 weeks exhibited a significant decrease in time to peak bioluminescence when compared to the time of implantation. Treatment with PTHrP 1-34 resulted in a more rapid time to peak bioluminescence at 4 weeks when compared to time-matched controls. There was a significant increase in both total and max fluorescence after treatment with PTHrP 1-34 at

both 2 and 4 weeks when compared to time-matched controls. Bars represent mean $\pm$ SD of 4–8 vossicles per treatment group.

Fig. 2.

Vossicle weights at baseline and after treatment with saline or PTHrP 1-34 for 4 weeks. For all figures going forward, “C” denotes a significant difference from the 2-week time point within the same treatment group. Control and PTHrP 1-34-treated mice had a significant increase in vossicle weight at 4 weeks when compared to baseline; however, no differences existed over time between treatment groups at 2 and 4 weeks. Bars represent mean $\pm$ SD of 4–8 vossicles per treatment group. See Figure 1 for the definition of additional symbols.

Fig. 3. Representative digital faxitron radiographs (a), individual vertebral body volumes (VBV, mm³) within the vertebral chain calculated from orthogonal digital radiographs (b), representative μCT scans (c), and μCT morphometric indices (d) at baseline and following therapy with saline or PTHrP 1-34 for 4 weeks. There was a significant increase in VBV after 2 weeks in both treatment groups that further increased after 4 weeks. Treatment with PTHrP 1-34 for 4 weeks resulted in the greatest increase in VBV. The radiographic assessment of individual VBVs within the vertebral chain were supported by μCT scans of one representative vossicle for each treatment group. Morphometric analysis of one representative vossicle for each treatment group by μCT revealed an increase in Tb.V/TV in PTHrP 1-34-treated vossicles at both 2 and 4 weeks. However, Tb.V/TV decreased in both treatment groups from 2 to 4 weeks. The increase in Tb.V/TV in PTHrP 1-34-treated vossicles was associated with an increase in Tb.N within the vertebrae and a decrease in Tb.Pf when compared to control vossicles at 2 and 4 weeks. For (a) and (c), the measurement bars equal 2 mm. For (b), bars represent mean ± SD of 4–8 vossicles per treatment group. See Figures 1 and 2 for the definition of symbols.

Fig. 4. Representative histologic sections (a) and the percentage of total bone (b), cortical bone (c), and trabecular bone (d) observed within the end vertebrae at baseline and after treatment with saline or PTHrP 1-34 for 4 weeks. The percentage of total bone was significantly increased in saline and PTHrP 1-34-treated vossicles at 2 and 4 weeks when compared to the time of implantation, with PTHrP 1-34 resulting in a greater amount of bone than time-matched controls. Evaluation of the cortical and trabecular bone compartments revealed a significant increase in the percentage of cortical bone within the vertebrae in both treatment groups at 2 and 4 weeks when compared to the time of implantation; however, no differences existed between treatment groups. The increase in the total amount of bone within the vertebrae in the PTHrP 1-34 treatment group at both 2 and 4 weeks when compared to the time of implantation and time-matched controls was due to an increase in trabecular bone mass. For (a), the measurement bar equals 500 μ m. For (b) – (d), bars

represent mean $\pm$ SD of 3–5 vossicles per treatment group. See Figures 1 and 2 for the definition of symbols and the results section for histologic descriptions.

Fig. 5.

Association between radiographic, histologic, and functional (BLI and FLI) indices. Both r and P values are listed. Solid circles indicate control vossicles and crosses indicate vossicles treated with PTHrP 1-34. Only those correlations that were statistically significant or approached statistical significance ($P < 0.10$) are shown. The correlation between total fluorescence and the percentage of cortical bone within the vertebrae was also shown. Key findings were that a positive but non-significant linear relationship existed between the percentage of total bone observed within the vertebrae histologically and total bioluminescence, total fluorescence, and max fluorescence. Closer examination revealed a positive linear relationship between the percentage of trabecular bone and total bioluminescence, total fluorescence, and max fluorescence that reached or approached statistical significance.