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Skeletal homeostasis in tissue-engineered bone

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Abstract

Tissue-engineering strategies to stimulate bone regeneration may offer an alternative approach to conventional orthopaedic and maxillofacial surgical therapies. Over the last decade, significant advances have been accomplished in developing biomimetic matrices, growth factors, cell transplantation and gene delivery therapeutics to support new bone growth. However, it is not known if tissue-engineered bone recapitulates the biology of normal skeletal tissue in response to physiologic cues. Here, we report that bone formed by the differentiation of transplanted murine bone marrow stromal cells (BMSCs) responds to a systemically delivered calciotropic hormone. Ectopic ossicles in mice exposed to catabolic doses of parathyroid hormone (PTH) had increased numbers of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts as compared to control mice. In contrast, treatment with anabolic doses of PTH promoted a marked increase in trabecular bone mass as analyzed by microcomputed tomography and histomorphometry. Our findings demonstrate that bone formed from transplanted BMSCs is responsive to normal physiologic signals, and can be augmented by the addition of a systemic anabolic agent. Because multiple and distinct ossicles can be generated in a single animal, this versatile system may be used to: (a) elucidate cellular/molecular mechanisms in bone regeneration; (b) study cell-to-cell interactions in the bone marrow microenvironment in health and disease; and (c) evaluate the efficacy of osteotropic agents that modulate bone turnover in vivo.

Keywords: Tissue engineering; Bone marrow stromal cells; Bone formation; Bone resorption; Parathyroid hormone; Mesenchymal stem cells

Introduction

Remarkable advances have been made to stimulate new bone growth via tissue-engineering strategies [26,30]. Biomimetic matrices, growth factors, cell transplantation and gene delivery therapeutics have all been utilized to optimize and support bone regeneration [8,16,25]. Despite recent developments, little attention has been given to determine if engineered bone responds to normal physiologic cues. Because the ultimate goal of bone tissue engineering is to recapitulate the structure and function of the native tissue it is designed to replace, it is critical to evaluate in well-characterized model systems whether this newly formed bone mimics normal skeletal tissue. Bone marrow stromal cells (BMSCs), also referred to as mesenchymal or skeletal stem cells, give rise to a variety of tissues of mesenchymal origin including bone [2,24]. Indeed, BMSC transplantation strategies have been applied to generate ectopic as well as orthotopic bone, the latter being a potential approach for skeletal regeneration procedures [4,13,17,23]. However, it is not known if tissue-engineered bone generated by osteogenic cell transplantation responds to systemic biologic factors in a similar manner as native bone.

The purpose of this study was to characterize the remodeling of tissue-engineered bone developed from the differentiation of transplanted murine BMSCs in response to catabolic and anabolic regimens of systemically administered recombinant human parathyroid hormone (PTH). As a major calciotropic peptide

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hormone, PTH plays a critical role in regulating extracellular calcium homeostasis. In addition, PTH is capable of stimulating both catabolic and anabolic actions in bone, depending on the dose and mode of administration [11]. Our findings demonstrate that tissue-engineered bone exposed to catabolic doses of PTH had increased osteoclastic activity, whereas treatment with anabolic doses resulted in increased trabecular bone mass. These studies suggest that bone developed from transplanted BMSCs responds to normal physiologic signals, and can be augmented by the addition of a systemic anabolic agent.

Materials and methods

Isolation of BMSCs. Four- to 8-week old C57BL/6 littermate mice were used to isolate BMSCs from the femoral, tibial and humeral cavities (six bones per animal) as previously described [15]. Fibroblast-like BMSCs are the predominant adherent cell type when bone marrow is cultured in vitro and they lack the basic characteristics of macrophages and endothelial cells [5,9]. First passage was carried out on day 10 when a confluent adherent cell layer was observed. Cultures were maintained for 5–7 days before cells were transplanted. All animal protocols were performed in compliance with the University of Michigan Committee for the Use and Care of Animals.

Surgical transplantation of BMSCs. Transplantation procedures were performed as previously described [15]. Pooled BMSCs (passage 2, $2.5–3 \times 10^6$ cells) were resuspended in 30–100 μL of growth medium and incorporated into gelatin sponges (Gelfoam®; Upjohn, Kalamazoo, MI) by capillary action. Immunocompromised 4- to 6-week-old male mice (N:NIH-bg-nu-xid) were utilized as transplant recipients. Following anesthesia with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (5 mg/kg), two midlongitudinal skin incisions of approximately 1 cm were made on the dorsal surface of each mouse. Subcutaneous pouches were formed by blunt dissection and four transplants were placed per animal.

PTH treatments. One week following transplantation, two groups of animals (8–10 animals/group) were given daily subcutaneous injections of either recombinant human PTH(1-34) (40 μg/kg) (Bachem, CA) or vehicle (0.9% sodium chloride) (Abbott, IL) for 21 days (anabolic regimen) [6]. Four weeks following transplantation, two groups of animals were injected subcutaneously with 50 μL of PTH(1-34) (2 μg) or vehicle every 6 h for 3 days (catabolic regimen) [28]. At sacrifice, which was 24 h (anabolic group) or 2 h (catabolic group) after the last injection, terminal serum samples were collected (Fig. 1A).

Radiography, microcomputed tomography (μCT), histology and histomorphometry. Radiographic analysis was performed using a microradiography apparatus (Faxitron X-ray Corporation, IL). For μCT analysis, specimens were scanned at 8.93 μm voxel resolution on an EVS Corp. μCT scanner. Morphometric measures were calculated using custom programs in IDL version 5.5. For histology, the ossicles were fixed in aqueous buffered zinc formalin for 24 h at 4 °C, decalcified 2 days in 10% EDTA, embedded in paraffin and 5-μm serial sections prepared and stained with hematoxylin/eosin. Sections from the catabolic group were also used for tartrate-resistant acid phosphatase (TRAP) staining using a leukocyte acid phosphatase kit from Sigma Diagnostics (St Louis, MO). Total bone area, trabecular bone area, cortical width, number of osteocytes/mm^2 and number of TRAP+ osteoclasts/mm were analyzed by computer-assisted histomorphometry using Image Pro Plus 4.1 (Media Cybernetics, Silver Spring, MD) and SPOT RT Diagnostic Inst. 3.0 (Sterling Heights, MI) software interfaced with a Nikon Eclipse E800 light/epifluorescent microscope (Nikon, Melville, NY) as previously described [19]. Histomorphometric determination of the cortical width was analyzed by carefully delineating the endocortical area without including any trabecular structure emerging from the endosteum.

Serum biochemistry. Serum calcium concentrations were determined by a colorimetric assay with cresophtalein complexone following the manufacturer's recommendations (Sigma Diagnostics).

Statistics. The data was analyzed using Student's *t* test with the Instat 2.1 biostatistics program (GraphPad Software, San Diego, CA). Data are presented as mean ± SEM.

Results

We characterized the responses of BMSC-derived bone to systemic PTH administered in a catabolic and anabolic regimen. Following transplantation into a host animal, BMSCs seeded in gelatin sponges, formed self-contained, highly vascularized, mineralized bone/bone marrow organs (Fig. 1B and C). These ectopic ossicles contained cortical and trabecular bone surrounding a normal marrow cavity with active hematopoiesis as previously described [15]. Because hematopoietic marrow functions as the major source of osteoclast precursors [29], we first investigated whether a catabolic regimen of PTH could induce the recruitment and activity of functional osteoclasts to the endosteal and

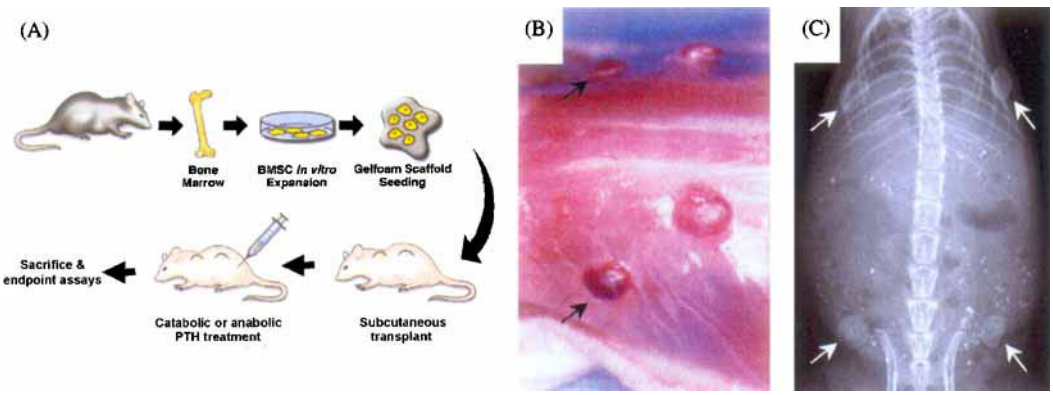

Fig. 1. Experimental design to characterize remodeling responses of tissue-engineered bone generated from transplanted murine bone marrow stromal cells (BMSCs). (A) Schematic of experimental procedures. Subcutaneous injections with either anabolic or catabolic doses of PTH(1-34) were initiated 1 or 4 weeks after cell transplantation, respectively, followed by sacrifice and endpoint assays. (B) Gross photograph of ossicles at sacrifice, and (C) radiograph of mineralized nodules (arrows) prior to their excision.

trabecular surfaces of the ossicles. Four weeks after cell transplantation, mice were administered either PTH (2 μg) or saline as a vehicle control, subcutaneously four times a day for 3 days, then sacrificed. Animals treated with PTH had significantly higher serum calcium concentrations than controls (12.12 ± 0.6 mg/dL (PTH) versus 9.93 ± 0.3 mg/dL (vehicle), $p < 0.02$, Fig. 2A). Under normal circumstances serum calcium levels are very similar in mice as compared to humans, ranging between 8.5 and 10.5 mg/dL. Interestingly, these similarities are also true in hypercalcemic states associated with diseases such as primary hyperparathyroidsm. In fact, a recent study shows that in a transgenic mouse model of human primary hyperparathyroidsm serum calcium levels were significantly elevated as compared to their wild-type littermates [12]. Serum calcium levels in the transgenic mice were between 11.4 and 12.45 mg/dL. In most patients with primary hyperparathyroidsm serum calcium levels are within 1 mg/dL above the upper limits of normal and usually <12 mg/dL [27]. Therefore, our results in PTH-treated mice are comparable to what it is clinically observed in patients with primary hyperparathyroidsm. The endosteal and trabecular surfaces of the ossicles also contained a considerably higher number of TRAP-positive osteoclast-like cells per millimeter of bone (3.93 ± 0.7 (PTH) versus

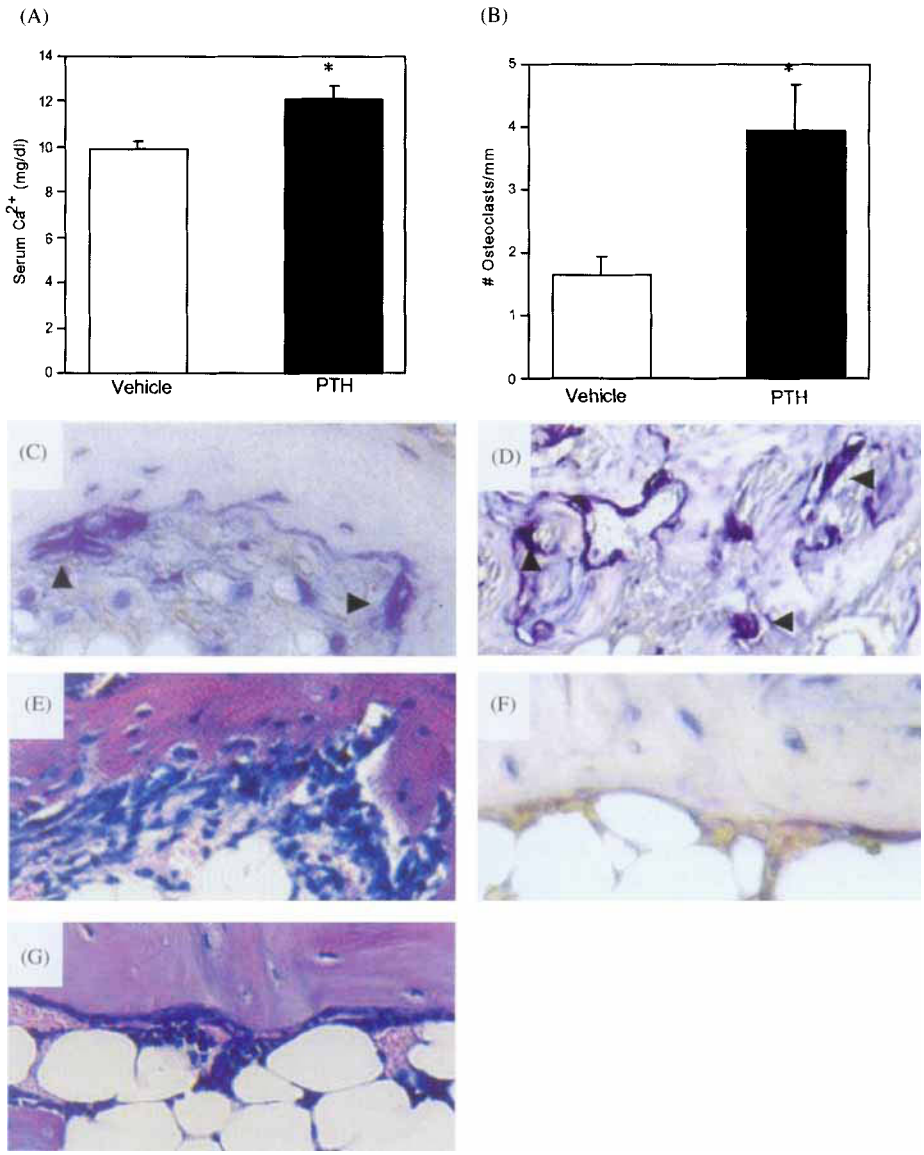

Fig. 2. PTH induced a catabolic response in ectopic tissue-engineered bone. (A) Calcemic responses in mice treated with catabolic doses of PTH ($n = 9$) or vehicle control ($n = 6$), $^*p < 0.02$. (B) Quantification of TRAP-positive osteoclast-like cells in ossicles exposed to catabolic doses of PTH ($n = 9$) versus controls ($n = 8$), $^*p < 0.01$. Osteoclast-like cells were found lining endosteal (C) and trabecular bone surfaces (D) (arrowheads) with higher cellularity adjacent to endosteal surfaces of PTH-treated ossicles (E) as compared to controls which demonstrated minimal TRAP positive stain (F) and less cellularity (G).

1.65 ± 0.3 (vehicle), $p < 0.01$, Fig. 2 B–D) and demonstrated an increased overall cellularity in close proximity to scalloped endosteal surfaces (Fig. 2E). In contrast, ossicles in vehicle-treated animals showed minimal TRAP-positive activity and cellularity (Fig. 2F and G). Interestingly, similar osteoclastic responses have been reported when localized catabolic doses of PTH are directly delivered to rat femurs [28]. Taken together, these data suggest that the hematopoietic marrow contained in the ectopic ossicles was able to support osteoclastic activity following systemic delivery of a potent stimulator of bone resorption such as PTH.

Next, the ability of the tissue-engineered bone to respond to an anabolic course of PTH was evaluated using an intermittent PTH dosing regimen. Daily subcutaneous injections of either PTH (40 μg/kg) or saline were initiated 1 week following BMSC transplantation and continued for 21 days, before the mice were sacrificed. Following surgical excision, microradiographic images of ossicles from PTH- and vehicle-treated mice were taken to determine their degree of mineralization. Representative ossicles derived from PTH-treated mice revealed a higher degree of radiopacity consistent with an increase in mineral content as compared to controls (Fig. 3A). These observations were further supported and quantified by histomorphometric analysis. Relative to the total area of the ossicle, the trabecular bone area was significantly higher in ossicles derived from PTH-treated mice ($37.04 \pm 7.4\%$ (PTH) versus $14.05 \pm 2.13\%$ (vehicle), $p < 0.05$, Fig. 3B and C). These positive changes in bone microarchitecture correlated with higher cellularity in the marrow cavity, a qualitative reduction in marrow fat content and a characteristic pattern of trabecular bone emerging from the cortex of the ossicles (Fig. 3B). Likewise, microcomputed tomographic (μCT) analysis revealed a marked increase in percent bone volume of the ossicles in mice treated with PTH ($48.2 \pm 1.9\%$ (PTH) versus $30 \pm 3.1\%$ (vehicle), $p < 0.01$, Fig. 3D and E). Although the number of osteocytes in the tissue-engineered cortical bone tended to increase with anabolic administration of PTH, it did not reach statistical significance. No significant differences between groups were observed in cortical bone width and in the overall dimensions of the ossicles (data not shown).

Discussion

The results of the present study demonstrate that ectopic tissue-engineered ossicles derived from transplanted BMSCs are responsive to catabolic and anabolic treatments with systemic PTH and thus may serve as an in vivo model to study skeletal homeostasis. One of the most significant findings of this study was the substantial increase in trabecular bone following treatment with anabolic doses of PTH as demonstrated by histomorphometric and μCT analyses. Currently, increasing interest exists for the use of anabolic agents in the treatment of metabolic bone diseases, particularly osteoporosis. In fact, among several potential anabolic agents, PTH is considered the most promising agent for the treatment of osteoporosis in women and men [7,21].

Our results also demonstrate that anabolic treatment with PTH positively affects growing skeletal structures like the ectopic ossicles. The basic mechanisms by which exogenous PTH exerts anabolic actions in bone are still poorly understood; however, these effects have been associated with increases in osteogenic cell proliferation and differentiation [22] and prolonged osteoblast survival [14]. Nishida et al. demonstrated that cultured BMSCs isolated from rodent long bones following a 3-week regimen with anabolic doses of PTH(1-34) generated more alkaline phosphatase positive-colony forming unit-fibroblastic colonies (CFU-F), suggesting that these cells were already committed to the osteoblastic lineage. These data correlated with significant increases in parameters related to bone formation including bone mineral density [22]. Therefore, PTH may be stimulating anabolic actions in bone in part by positively regulating the pool of osteoprogenitor cells available to differentiate into bone-forming cells. Interestingly, recent studies on the effects of PTH in developing bone suggests that PTH is also critical to achieve normal fetal endochondral bone formation by affecting cartilage mineralization and osteoblast proliferation, differentiation and survival in the endosteum and primary spongiosa [6,20].

The substantial increase in trabecular bone in the ossicles indicates that ectopic tissue-engineered bone may also provide a powerful, new strategy to test skeletal pharmacotherapeutic agents designed to improve bone quality. Skeletal homeostasis in response to biologic factors is usually studied in model systems that include normal, gonadectomized, transgenic animals, and more recently, transplanted bone fragments [1,3,10,18]. Despite their valuable role, they have limited application to bone tissue engineering because they evaluate remodeling responses in fully developed native bone. The ectopic tissue-engineered bone model system offers the advantage that several and distinct ossicles can be generated in a single host and individual ossicles can be retrieved at different time points without sacrificing the animal.

This model system goes beyond the application of cell transplantation systems for skeletal regeneration purposes. By demonstrating that the newly formed bone behaves as normal skeletal tissue, cell-based bone tissue-engineering strategies may be utilized in conjunction with anabolic agents, such as PTH, to amplify and further optimize de novo bone formation in regenerating skeletal sites. In fact, adding a physiologic stimulus to

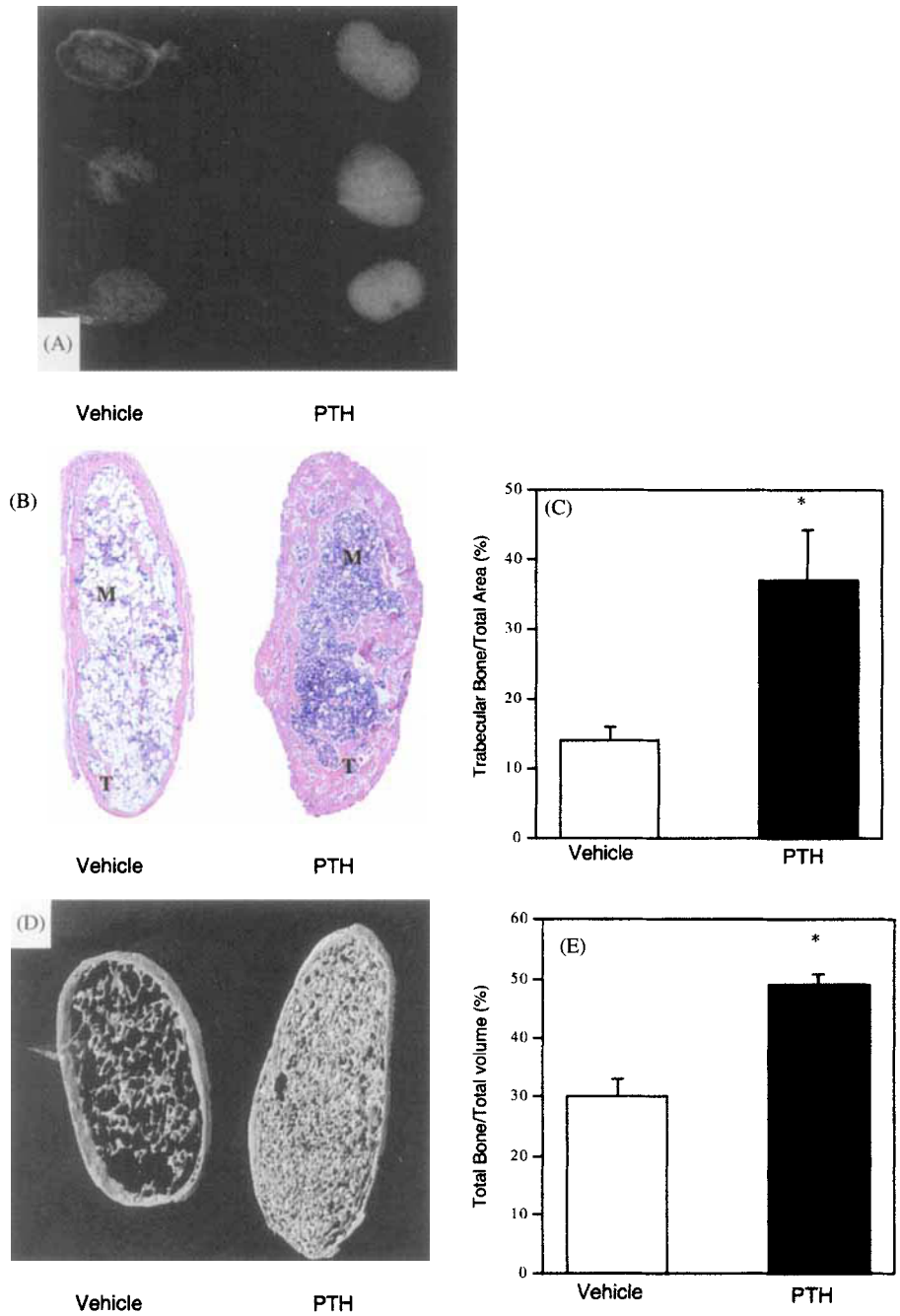

Fig. 3. PTH induced an anabolic response in ectopic tissue-engineered bone. (A) Microradiographic image of three ossicles exposed to vehicle (left) or PTH treatment (right). (B) H & E stained histological sections of newly formed bone derived from BMSC transplantation. Note the increase in trabecular bone (T) and marrow cellularity (M) in the ossicle exposed to anabolic doses of PTH (right). (C) Quantification of trabecular bone area per total area of ossicle by histomorphometric analysis in representative implants from PTH- ($n = 7$) and vehicle control-treated animals ($n = 4$), $^{*}p < 0.05$. (D) μCT images of representative ossicles derived from animals treated with vehicle control (left) or PTH (right). (E) Quantification of the total bone volume relative to the total volume of the implant by μCT analysis in representative ossicles derived from PTH- ($n = 3$) and vehicle control-treated animals ($n = 3$), $^{*}p < 0.01$.

enhance the regenerative capacity of engineered bone may result in bone that more effectively mimics the native tissue.

Moreover, this versatile system may assist a wide range of scientists from academia to the pharmaceutical industry to better understand in vivo bone physiology in health and disease and design studies to: (a) elucidate cellular/molecular mechanisms in bone regeneration and homeostasis (i.e., signaling pathways); (b) study normal or aberrant cell-to-cell interactions in the bone marrow microenvironment (i.e., tumor cell/bone cell interactions); and (c) evaluate the efficacy of

pharmacotherapeutic agents that modulate bone turnover in vivo.

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