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Immunohistochemical and morphometric assessment on the biological function and vascular endothelial cells in the initial process of cortical porosity in mice with PTH administration

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Running Headline/Short Title: Cortical porosity in PTH administered mice

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Abstract

To clarify the cellular mechanism of cortical porosity induced by intermittent PTH administration, we examined the femoral cortical bone of mice that received 40μg/kg/day (four times a day) hPTH [1-34]. The PTH-driven cortical porosity initiated from the metaphyseal region and chronologically expanded toward the diaphysis. ALP-positive osteoblasts in the control mice covered the cortical surface, and endomucin-positive blood vessels were distant from these osteoblasts. In PTH-administered mice, endomucin-reactive blood vessels with TRAP-positive penetrated the ALP-positive osteoblast layer, invading the cortical bone. Statistically, the distance between endomucin-positive blood vessels and the cortical bone surface abated after PTH administration. TEM observation demonstrated that vascular endothelial cells often pass through the flattened osteoblast layer and accompanied osteoclasts in the deep region of the cortical bone. The cell layers covering mature osteoblasts thickened with PTH administration and exhibited ALP, αSMA, VCAM1, and RANKL. Within these cell layers, osteoclasts were found near endomucin-reactive blood vessels. In PTH-administered femora, osteocytes secreted Dkk1, a Wnt inhibitor that affects angiogenesis, and blood vessels exhibited PLVAP, an angiogenic molecule. In summary, endomucin-positive blood vessels, when accompanied by osteoclasts in the ALP/αSMA/VCAM1/RANKL-reactive osteoblastic

cell layers, invade the cortical bone, potentially due to the action of osteocyte-derived molecules

such as DKK1.

Introduction

Contemporary comprehension of the mechanisms underlying bone formation stimulated by drug treatments for osteoporosis can be divided into two principal histological processes: remodeling-based and modeling-based bone formation [1]. Remodeling-based bone formation follows osteoclast bone resorption, whereas modeling-based bone formation involves independent occurrence of bone formation and resorption. Among these, teriparatide, a synthetic human parathyroid hormone (PTH) analog, is a commonly used agent for investigating remodeling-based bone formation. PTH-driven bone anabolism is predicated by cell coupling between osteoclasts and osteoblasts. This finding is evidenced by animal models featuring PTH stimulating preosteoblasts and osteoblastic bone formation in normal mice, without facilitating osteoblasts in forming new bone in the absence of bone-resorbing osteoclasts in *c-fos*^{-/-} mice [2].

We previously reported that frequent PTH administration results in remodeling-based bone formation characterized by numerous thin metaphyseal trabeculae surrounded by thick preosteoblastic cell layers with several osteoclasts [3]. Conversely, low frequent PTH administration induced remodeling-based and modeling-based bone formation with relatively thick trabeculae. Therefore, high PTH administration frequency may stimulate cell coupling of osteoclasts and osteoblasts, accelerating the remodeling-based bone formation of metaphyseal trabeculae.

Contrasting metaphyseal trabeculae, Burr's team discovered that daily hPTH [1-34] administration triggered intracortical remodeling in the midshaft of the rabbit tibia, increasing cortical porosity [4]. Subsequently, these results raise concerns that intermittent PTH treatment for osteoporosis in adult female cynomolgus monkeys may compromise cortical bone strength due to porosity induction [5]. Clinical studies have reported cortical porosity following daily teriparatide administration, corroborating these findings [6,7].

The specific site of cortical bone susceptibility to teriparatide-induced porosity has been identified in the region close to the endocortical surface [5, 8]. Furthermore, PTH administration frequency and dosage regimens have been implicated in cortical porosity. Several investigators explored the effects of intermittent teriparatide administration, discovering that higher daily doses increased cortical porosity in rabbits, as opposed to weekly dosing [9, 10]. Utilizing HR-pQCT, Hansen *et al.* reported that a regimen of PTH administration had deleterious effects on cortical bone microarchitecture and strength in postmenopausal women with osteoporosis [6].

Many researchers have used mouse models to inhibit or overexpress osteocyte-derived sclerostin, a Wnt inhibitor, postulating that cell coupling between osteoclasts, osteoblasts, and osteocytes mediates PTH-driven anabolic effects in spongy bone and porosity in cortical bone [11-16]. Subsequent studies have revealed the expression of the PTH receptor in osteocytes [12-16]. Notably, blocking sclerostin in the bone anabolism with sclerostin antibodies has resulted in the development of romosozumab, a drug that can induce modeling-based bone formation in

osteoporotic patients [17, 18]. Therefore, sclerostin secreted by osteocytes may be involved in modeling-based bone formation rather than remodeling-based bone formation. Nonetheless, osteocytes may influence PTH-driven cortical porosity through mechanisms beyond sclerostin.

Meanwhile, we have previously highlighted an increase in endomucin[high]-positive bone-specific blood vessels, surrounded by α smooth muscle actin (αSMA)-positive cells after PTH administration. αSMA is recognized for its expressed perivascular and undifferentiated mesenchymal cells, suggesting interactions with vascular endothelial cells [19]. These αSMA-positive cells extend out from blood vessels and express alkaline phosphatase and c-kit, establishing their potential to differentiate into osteogenic and vascular endothelial/perivascular cells [20, 21]. Thus, PTH affects osteoclasts, osteoblasts, and other cell types, including osteocytes and blood vessels. This characteristic may be pivotal in not only bone anabolism of spongy bone but also cortical porosity.

Taken together, it is imperative to consider osteoclasts, osteoblasts, and the involvement of osteocytic network and bone-specific blood vessels to elucidate the cellular mechanism behind cortical porosity. In addition, the histological degree of the cortical porosity must be different from the metaphyses to the diaphysis of long bones. This study identified three distinct regions for cortical porosity observation: zonation of regions close to the growth plate, intermediate regions between metaphyses and diaphysis, and regions towards diaphysis. Therefore, we aimed to histologically investigate the spatiotemporal distribution of osteoblasts,

osteoclasts, and bone-specific blood vessels in these three regions throughout PTH treatment.

Additionally, we sought to elucidate the cellular interactions and inducing factors that

contribute to cortical bone porosity through histological analysis.

Materials and Methods

Animals

Six-week-old male C57BL/6J mice (n = 60, Japan CLEA, Tokyo, Japan) were handled per

Japanese Act on the Welfare and Management of Animals and Hokkaido University's guidelines

for animal care and research use (approved study protocol #20-0019). The mice were divided

into two groups: a control group receiving vehicle only (Control group, 0.9% saline, n = 6) and

a PTH group receiving hPTH [1-34] (Sigma-Aldrich Co., LLC., St. Louis, MO) at a dose of

40μg/kg/day, administered four times per day (PTH group, n = 6 for 1, 3, 7, 14, 21 days). The

dosage and frequency of PTH administration were decided based on the results of a previous

study.[3]

Specimen Preparation

The left hind legs of mice were ligated at the femoral region under anesthesia using an

intraperitoneal injection of an anesthetic agent combination (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol). Left tibiae were extracted for subsequent RNA sequencing and real-time PCR analyses. Femora were fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) via the left ventricle. The right femora were removed and immersed in the same fixative for 24 hr. After obtaining micro-CT images, the femora underwent decalcification with 10% ethylenediaminetetraacetic disodium salt (EDTA-2Na) for two months at 4C before paraffin embedding. Sagittal sections, 5 μm thick, were cut parallel to the longitudinal axis of the bone. Left femora were longitudinally divided. One piece was decalcified with a 4.13% EDTA-2NA solution before epoxy resin embedding. The other was post-fixed with 1% osmium tetraoxide in a 0.1 M cacodylate buffer for 4 hr at 4C, dehydrated in ascending acetone solutions, and embedded in epoxy resin (Epon 812, Taab, Berkshire, UK). Histochemical staining sections were photographed using a Nikon Eclipse E800 microscope (Nikon Instruments Inc., Tokyo, Japan), and light microscopic images were captured with a digital camera (Nikon DXM1200C, Nikon Instruments Inc., Tokyo, Japan).

Micro-CT Analysis

Micro-CT images of the right femora's sagittal section, spanning from the distal epiphysis to 1.6 cm distally, were obtained using a micro-CT (CosmoScan Fx, Rigaku Corporation, Tokyo, Japan) with a 90 kV tube voltage and 88 μA tube current (FOV 10 mm) following our recent

report [22].

Selection of Different Regions of Interest (ROI) in the Cortical Bone

Posterior femoral cortical bones were categorized into three regions of interest (ROIs): the metaphyseal region extending from 0.95 mm proximal to the growth plate to 0.7 mm towards the distal region (Zone 1), the region from 1.4 to 2.1 mm (Zone 2), and the diaphyseal region from 2.1 to 2.8 mm (Zone 3) (Fig. 1A).

Immunohistochemistry for α-Smooth Muscle Actin (αSMA), Vascular Cell Adhesion Molecule-1 (VCAM1), Sclerostin, Dickkopf1 (Dkk1), and PLVAP

Dewaxed paraffin sections were processed for αSMA, VCAM1, sclerostin, Dkk1, and PLVAP, as previously described [23]. In brief, the sections were immersed in 0.3% H_2O_2 in phosphate-buffered saline (PBS) for 30 min to block endogenous peroxidase. Next, 1% bovine serum albumin (Serologicals Proteins Inc., Kankakee, IL) in PBS (1% BSA-PBS) was applied to the sections for 20 min to minimize nonspecific binding. The sections were incubated with mouse monoclonal antiserum to αSMA (MAB1420, R&G systems Inc., Minneapolis, MN) at a 1:600 dilution, goat polyclonal antiserum against VCAM1 (AF643, R&G systems Inc., Minneapolis, MN) at a 1:100 dilution, goat polyclonal antiserum against sclerostin (AF1589, R&G systems Inc., Minneapolis, MN) at a 1:50 dilution, goat polyclonal antiserum against Dkk1 (AF1765,

R&G systems Inc., Minneapolis, MN) at a 1:100 dilution, or rat polyclonal antiserum against

PLVAP (ab27853, Abcam PLC, Cambridge, UK) at a 1:100 dilution at room temperature (RT)

for 2 hr. The sections reacted with the primary antiserum against αSMA were incubated with

horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (40267F, Thermo Fisher

Scientific Inc., Waltham, MA) at a dilution of 1:100 for 1 hr at RT. The sections reacted with

antisera to VCAM1, sclerostin, Dkk1, or PLVAP were incubated with HRP-conjugated rabbit

anti-goat IgG (40267F, American Qualex International, Inc., Clemente, CA) or HRP-conjugated

rabbit anti-rat IgG (A18921, Thermo Fisher Scientific Inc. Waltham, MA). We have optimized

all antibody dilutions specifically for use in this study. Diaminobenzidine tetrahydrochloride

(DAB) was used as a substrate to visualize all HRP-conjugated immunoreactions. All sections

were counterstained with methyl green and examined under a light microscope. Negative

control experiments utilized normal mouse, goat, and rat sera as primary antibodies instead of

antisera against the specific antigens (data not shown).

Double Detection of Endomucin/TRAP, Endomucin/ALP, PDGFbb/Endomucin, and αSMA/Endomucin

For endomucin/TRAP and endomucin/ALP double detection, the sections were treated with

polyclonal rat IgG against mouse endomucin (sc-65495, Santa Cruz Biotechnology, Inc., Dallas,

TX) and subsequent HRP-conjugated rabbit anti-rat IgG for DAB visualization, as previously

described. For TRAP detection, immunostained sections were incubated in a mixture of 2.5 mg

of naphthol AS-BI phosphate (Sigma-Aldrich Co., LLC., St. Louis, MO), 18 mg of red violet

LB salt (Sigma-Aldrich Co., LLC., St. Louis, MO), and 100 mM L (+) tartaric acid (0.76 g,

Nacalai Tesque, Inc., Kyoto, Japan) diluted in 30 mL of a 0.1 M sodium acetate buffer (pH 5.0)

for 15 min at 37C, as reported previously [24]. Similar to ALP immunoreactivity detection,

sections reacted with the endomucin antibody were incubated with rabbit sera and tissue

nonspecific alkaline phosphatase (ALP) [25, 26] at RT for 2 hr, then reacted with ALP-conjugated

goat anti-rabbit IgG (111-055-003, Jackson Immune Research Laboratories Inc., West Grove,

PA). ALP histochemistry was performed as described by Zhao et al. [20]. In short, sections were

incubated in a mixture of 2.5 mg of naphthol AS-BI phosphate (Sigma-Aldrich Co., LLC., St.

Louis, MO) and 18 mg of fast blue BB salt (Sigma-Aldrich Co., LLC., St. Louis, MO) diluted

in 30 mL of a 0.1 M Tris-HCl buffer (pH 8.5) for 15 min at 37C. Regarding PDGFbb/endomucin

and αSMA/endomucin double staining, the sections were incubated with polyclonal rabbit IgG

against mouse PDGFbb (ab23914, Abcam PLC., Cambridge, UK) at a 1:300 dilution or mouse

monoclonal antiserum to αSMA (MAB1420, R&G systems Inc., Minneapolis, MN) at a 1:600

dilution at RT for 2 hrs. Next, sections were reacted with HRP-conjugated swine anti-rabbit IgG

(P0399, Agilent Technologies Inc., Santa Clara, CA) or HRP-conjugated rabbit anti-mouse IgG

(61-6520, Thermo Fisher Scientific Inc., Waltham, MA) before DAB visualization. Treated

sections were incubated with polyclonal rat antibody to endomucin (Santa Cruz Biotechnology,

Inc., Dallas TX) and then with ALP-conjugated goat anti-rat IgG (112-055-003, Jackson Immune Research Laboratories Inc., West Grove, PA). ALP enzyme reaction for visualizing endomucin immunoreactivity was performed, as previously mentioned. We have optimized all antibody dilutions specifically for use in this study. In addition to those already presented, negative control experiments involved the use of normal rabbit and rat sera as primary antibodies instead of antisera against the specific antigens (data not shown).

Von Kossa Staining

Epoxy resin sections from undecalcified specimens were incubated with an aqueous solution of silver nitrate until the dark brown/black staining of bone tissue was visible under a light microscope [27].

Transmission Electron Microscopy Studies

Semi-thin sections of epoxy resin-embedded specimens were cut with an ultramicrotome (Sorvall MT-5000; Ivan Sorvall, Inc., Norwalk, CT), stained with toluidine blue, and observed with a Nikon Eclipse E800 microscope (Nikon Instruments Inc., Tokyo, Japan). Ultra-thin sections obtained with an ultramicrotome were stained with uranyl acetate and lead citrate. These specimens were subjected to transmission electron microscopic (TEM) observation (Hitachi H-7800, Hitachi Co., Tokyo, Japan) at 80 kV.

Quantification of the Distance Between Blood Vessels and Endosteal Bone Surface and osteoclast numbers in Assumed Bone Areas Posterior

Osteoclast numbers were counted in assumed boxed areas 700 μm in length and 100 μm in width (Fig 1B). We regarded TRAP-positive cells with more than two nuclei as osteoclasts. As depicted in Fig. 1C, the distance between the cortical bone surface and endomucin-positive blood vessels in each zone was calculated as the index obtained by dividing the area encompassing endomucin-positive blood vessels and the cortical bone surface by the cortical bone surface length.

RNA Sequencing Analysis

The total RNA obtained from the left tibiae of the control and PTH groups at 7 days (1 week) was employed for RNA sequencing analysis (n = 3 per group). The base sequences of the library-adjusted sample were collected (acquired read length: 150 bp × 2 paired ends) using the next-generation sequencer NovaSeq 6000 (Illumina, Inc., San Diego, CA), the library adjustment reagents NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490; New England Biolabs Japan Inc., Tokyo, Japan), and NEBNext Ultra TMII Directional RNA Library Prep Kit (E7760; New England Biolabs Japan Inc., Tokyo, Japan). The sequence reads were trimmed using Trimmomatic ver. 0.38 and mapped to the mouse reference genome (mm10)

using HISAT2 ver. 2.1.0. The raw reads mapped to known exon regions, FRKM, and TPM were

calculated using featureCounts ver. 1.6.3 to quantify gene expression. Differentially expressed

genes in the control and PTH-administered groups were extracted using DeSeq2 (ver. 1.24.0)

to create a heatmap.

The Quantitative Analysis of Gene Expression Through RT-qPCR

The cortical bones of the control and PTH groups were used for real-time PCR on 7 days (1

week) and frozen in liquid nitrogen. The samples were homogenized by adding 10 mL/g of

TRIzol reagent (Life Technologies Co., Carlsbad, CA). Then, 2 mL of chloroform was added

to 10 mL of TRIzol solution, and the mixture was centrifuged at 15,000 rpm for 5 min at 4C to

remove small debris. The supernatant was then transferred to a new tube, which was vortexed

for 15 s after adding 2 mL of chloroform. The lysate was then transferred to a new tube and

incubated for 5 min at RT. After phase separation, the aqueous phase containing the RNA was

transferred to a new tube. Then, RNA was precipitated by adding 5 mL of isopropyl alcohol per

10 mL of TRIzol reagent. After 10 min of incubation at RT, the mixture was centrifuged for 60

min at 15,000 rpm at 4C. The resulting RNA pellet was washed with 1 mL of 75% ethanol and

briefly air-dried. The RNA was then dissolved in 30 μL DEPC-treated water. First-stranded

cDNA was synthesized from the obtained total RNA with the SuperScript VlLO cDNA

Synthesis Kit (Life Technologies Co., Carlsbad, CA). The expression levels of various genes

relative to those of *Gapdh* were comparatively quantified via the ΔΔCt method in a quantitative

PCR System (StepOne; Thermo Fisher Scientific Inc., Waltham, MA) to determine *Acta2*

(αSma), Emcn, Tnfsf11 (Rankl), Pdgfb, Sost, Dkk1, Ckap4, and *Plvap* expression. The TaqMan

probes (Applied Biosystems, Waltham, MA) used for the analysis are shown in Table 1.

Statistical analysis

All statistical analyses were assessed through One-way ANOVA followed by Student's *t*-test

with Bellcurve for Excel ver. 4.04 (Social Survey Research Information Co., Ltd. Tokyo, Japan).

All values are presented as the mean ± SD. Values of $P < 0.05$ were considered significant.

Results

Micro-CT and histological images of PTH-induced cortical porosity

 Under 40 μg/kg/day of hPTH [1-34] four times a day, micro-CT images of mouse femora

chronologically demonstrated a marked increase in the trabecular bone beneath the growth plate

and the cortical bone transformation into a porous, trabecular bone-like structure (Fig. 2A)

From 1 week after PTH administration, initial cortical bone porosity became visible (Fig. 2A

IV-VI). The sagittal view of the cortical bone on the femoral posterior side revealed the porous

structure from the distal (metaphyseal) to the proximal (diaphyseal) regions as the experimental

period progressed (Fig. 2A I-XII). When observed horizontally, these pores formed the endosteal side towards the mid-region of the cortical bone (Fig. 2A i-vi). Consistent with the micro-CT images, histological sections indicated a chronological increase in trabecular bone volume and porous structure in PTH-administered femora (Fig. 2B). Thus, PTH-induced cortical porosity appeared chronologically from metaphyseal to diaphyseal regions in sagittal sections and from the endosteal to mid-region in horizontal sections.

Double detection of endomucin/TRAP and endomucin/ALP in the cortical bone

Next, we examined the dual staining for the endothelial cell marker endomucin and the osteoclast marker TRAP in three distinct zones (1-3) after 1 week: the region close to the growth plate (Zone 1), the intermediate regions between metaphyses and diaphysis (Zone 2), and the region towards diaphysis (Zone 3) (Fig. 3). In Zone 1, the cortical bone in control mice predominantly possessed several endomucin-positive blood vessels between the cortical bone and trabeculae, covered by ALP-reactive osteoblasts (Fig. 3D). When observing towards the diaphysis (Zones 2 and 3), ALP-reactive osteoblasts were located on the smooth endosteal bone surface (Fig. 3E, F).

Endomucin-positive blood vessels were close but never penetrated the ALP-reactive osteoblastic layers to reach the bone surfaces (Fig. 3E, F). There were few TRAP-positive osteoclasts on the endosteal bone surface in Zones 1-3 (Fig. 3A-C). In contrast, in the PTH-

administered group, Zone 1 exhibited many osteoclasts and thick ALP-reactive osteoblastic layers covering the bone surface (Fig. 4A, D). In Zones 2 and 3, many blood vessels with small diameters and TRAP-positive osteoclasts tended to accumulate on the rough endosteal bone surface (Fig. 4B, C). ALP-positive osteoblastic layers developed, though they were not as thick as those in Zone 1 (Fig. 4E, F). Some TRAP-positive osteoclasts and endomucin-positive blood vessels invaded the cortical bone's superficial layer (Fig. 4B, C).

Distance of blood vessels from the endosteal bone surface and osteoclast numbers in the assumed boxed areas

We measured the distance between blood vessels and the endosteal bone surface and quantified TRAP-positive osteoclast numbers in the assumed boxed areas with a 700 μm length and 100 μm width (See Fig. 1B, C, and Materials and Methods). The distance between blood vessels and the bone surfaces significantly decreased in the PTH groups compared with the control groups from 1 day to 1 week in Zones 1-3 (Fig. 5A-C). In contrast, statistical analyses revealed increased osteoclast numbers in the PTH-administered groups from 1 day to 1 week relative to the control groups (Fig. 5D-F). Therefore, it seems likely that endomucin-positive blood vessels and TRAP-positive osteoclasts gradually approached the endosteal bone surfaces.

Fine structure of blood vessels invading the cortical bone

High-resolution semi-thin sections and TEM observations revealed flattened osteoblasts and osteoclasts on bone surfaces and blood vessels near bone surfaces after 1 week in the PTH group (Fig. 6A-D). When observing blood vessels invading the cortical bone's superficial layer, the bone matrix close to the tips of invading blood vessels was well-calcified (Fig. 6E, F). However, TEM observations demonstrated that vascular endothelial cells and osteoclasts accompanied one another on the tip of invading blood vessels (Fig. 6G).

Histological characterization of osteoblastic layers over mature osteoblasts in the PTH-administered groups

As previously reported [20], we have examined whether the osteoblastic cell layer would exhibit αSMA-reactivity in specimens 1 week after PTH administration (Fig. 7). The control specimens showed only αSMA-positive cells (identical to arterioles and venules) in the periosteum but hardly any on endosteal bone surfaces (Fig. 7A-C). Conversely, in the PTH groups, Zone 1 displayed αSMA-positive thick cell layers identical to ALP-reactive osteoblastic layers surrounding bone surfaces; Zone 2 exhibited thin αSMA-positive cell layers (Fig. 7E-G). Next, we double-stained αSMA and endomucin localized blood vessels invading the cortical bone underneath the αSMA-positive cell layer in the PTH group (Fig. 7H). However, no αSMA-positive cell layer was seen in the control group (Fig. 7D). Consistently, real-time PCR verified the increased expression of *Acta2 (αSma)* and *Emcn (Endomucin)* in PTH groups (Fig. 7I, J).

Furthermore, in the PTH group, VCAM1 immunoreactivity was observed in the corresponding osteoblastic cell layer, which could be seen as a thick cell layer in Zone 1 and a thin layer in Zone 2 (Fig. 8A-D). In addition, the double detection of RANKL/TRAP identified many osteoclasts in the osteoblastic layers with RANKL reactivity in Zones 1 and 2 of the PTH group (Fig. 8E-H). We examined the double detection of PDGFbb that reportedly modulated angiogenesis and endomucin in osteoclastic cells [28]. PDGFbb-reactive osteoclastic cells were near endomucin-positive blood vessels, as observed in Zones 1 and 2 of the PTH groups, but not in the control group (Fig. 8I-L). Consistent with RANKL and PDGFbb histochemistry, real-time PCR indicated elevated *Tnfsf11 (Rankl)* and *Pdgfb* expression (Fig. 8M, N). PTH-administered femora displayed ALP-reactive osteoblastic cell layers also possessing αSMA, VCAM1, and RANKL. The close localization of PDGFbb-positive osteoclasts and endomucin-reactive blood vessels was observed among these cell layers. This finding implicates the possibility that endomucin-reactive blood vessels and osteoclasts could migrate through VCAM1-positive/RANKL-reactive osteoblastic cell layers to reach the endosteal bone surface.

DKK1, Ckap4, and Plvap immunolocalization and expression in PTH-administered femora

We turned our attention to genes expressed by osteocytes, as we considered them potential candidates for vascular invasion into cortical bone, given their predominant presence inside

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cortical bone. Our investigation involved the examination of molecules secreted by angiogenetic factors and osteocyte-derive molecules through RNA sequencing (Fig. 9). Heat map analysis revealed the elevated expression of many angiogenic factors, including *Hif1α, Pdgfb, Vash2, Ephb4, Efnb2, Ramp3, Ephb2, Pik3r3, Srpx2, Aplnr, Gas1, Cd276, Dkk1* (a Wnt inhibitor and angiogenic activator), *Ckap4* (a Dkk1 receptor), and *Plvap* (plasmalemma vesicle-associated protein, an angiogenetic factor) and osteocyte-derived molecules, such as *Dmp1, Phex, Tnfsf11,* and *Dkk1*, in the PTH group (Fig. 9A, B). Among them, we focused on Dkk1, Ckap4, and Plvap. Sclerostin was observed in osteocytes and their canaliculi in the control and PTH groups, with no significant difference in the *Sost* expression (Fig. 9C, D, I). However, Dkk1 gene expression was significantly elevated after PTH administration, and intense immunoreactivity was observed in osteocytes in the PTH group (Fig. 9E, F, J). Real-time PCR verified the elevated *Ckap4* expression (Fig. 9K), and blood vessels close to and invading the cortical bone definitively showed Plvap immunoreactivity in the PTH group but not in the control group (Fig. 9G, H). Consistently, *Plvap* expression was significantly elevated (Fig. 9L).

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Discussion

In our current study using a mouse model, we observed that cortical porosity progresses from the metaphyseal region to the diaphyseal region in sagittal sections and the endosteal bone surface into the mid-region of the cortical bone in transverse sections. PTH administration caused blood vessels to migrate towards the endosteal bone surface, passing through the ALP-positive osteoblastic layer. These osteoblastic layers were thick in the metaphyseal region but thin in the diaphyseal region. Furthermore, PTH administration resulted in not only blood vessels' migration on the endosteal bone surface but also the appearance of osteoclasts in the close vicinity of the invading blood vessels. We postulate that the closely coordinated migration of blood vessels and osteoclasts toward the cortical bone surface is essential during the early stages of cortical porosity in our mouse models.

We hypothesized that while blood vessels approached the osteoblastic layer covering mature osteoblasts and penetrated the cortical bone, the ALP-positive osteoblastic layer is pivotal in endomucin-positive blood vessels migration. Blood vessels in the control group maintained a certain distance from the bone surface, and there were no signs of blood vessels penetrating the cortical bone. The endosteal surface of the mouse femur observed in this study gradually underwent bone formation through modeling [29]. Therefore, osteoclasts are nearly absent under normal conditions.

In contrast, the ALP-positive thick osteoblastic layer in PTH-administered mice exhibited

characteristics such as αSMA and VCAM1 positivity. αSMA is a marker for perivascular and undifferentiated mesenchymal cells [19], while VCAM1 is an adhesion molecule expressed in endothelial, undifferentiated mesenchymal, and bone marrow stromal cells [30]. Therefore, vascular endothelial cells could migrate toward endosteal bone surfaces using VCAM1-positive osteoblastic cell layers as a scaffold. On the other hand, the αSMA-positive osteoblastic cell layer implies that these cells may still possess perivascular cell characteristics, suggesting a potential interaction with vascular endothelial cells.

Our previous reports indicated that PTH-treated mice exhibited increased osteoblastic progenitor proliferation in the metaphyses of long bones, vascular lumen diameter, and vessel numbers. In addition, many αSMA-positive cells extended into the endomucin-positive blood vessels and the surrounding tissue [20, 21]. These αSMA-positive cells, which also exhibit ALP and VCAM1 positivity, are postulated to be undifferentiated mesenchymal cells capable of differentiating into perivascular cells and osteoblast progenitors. Therefore, the ALP/αSMA/VCAM1-positive cells in this study, spreading gradually from the metaphyseal to diaphyseal regions, are likely to induce cortical porosity.

Many TRAP-positive osteoclasts in PTH-treated mice were included in the thick osteoblastic layer covering the endosteal bone surface. Statistical analysis of the distance between blood vessels and bone surfaces strongly indicates that endomucin-positive blood vessels gradually approached the endosteal bone surface after PTH administration. Additionally,

these endomucin-positive blood vessels were near osteoclasts producing PDGF-bb, as shown

in Figure 8. On the other hand, the femora's endosteal bone surface of growing mice is a bone

modeling region where continuous calcein labeling has been observed [29].

The many osteoclasts induced by PTH administration are likely due to the

osteoclastogenesis potential of the osteoblastic cell layer that is positive not only for

ALP/αSMA/VCAM1 but also for RANKL. Although these RANKL-positive osteoblastic cells

thin out when approaching the diaphyseal region, a niche for osteoclast induction in situ is

needed. Thus, RANKL-positive osteoblastic layers covering the endosteal bone surface may

induce osteoclasts, keeping the close proximity to blood vessels by mediating PDGFbb

synthesis. Notably, the osteoclasts accompanied by blood vessels would be crucial for

penetrating the cortical bone interior.

In this study, von Kossa staining revealed that the tips of blood vessels invading the deep

cortical bone are calcified. It is challenging for the endothelial cells in the blood vessels to

dissolve the calcified matrix. However, TEM observations revealed osteoclasts lined up

alongside invading endothelial cells, suggesting that osteoclasts dissolved the calcified bone

matrix. As such, osteoclasts participating in migration and penetration in the cortical bone

interior seem intrinsic to the initial cortical porosity process.

One may wonder what prompts blood vessels and osteoclasts to invade the cortical bone

interior. The osteocyte network is a potential candidate since osteocytes are predominately

embedded in bone. RNA sequencing revealed that PTH administration up-regulated *Hif1α, Pdgfb, Vash2, Ephb4, Efnb2, Ramp3, Ephb2, Pik3r3, Srpx2, Aplnr, Gas1, Cd276,* and *Dkk1*. Among these genes, we noted that *Dkk1* is an osteocyte-derived molecule, which was reportedly as an inhibitor of Wnt signaling and an angiogenesis activator [31-33]. Since *Ckap4* (a Dkk1 receptor) [34, 35] and *Plvap* (plasmalemma vesicle-associated protein, an angiogenetic factor) [36] expressions were also elevated, we postulated that osteocyte-derived molecules, such as Dkk1, affected vascular endothelial cells to induce angiogenesis. This reaction potentially enables invasion into the cortical bone with osteoclasts in the PTH group.

The human cortical bone comprises many osteons, a concentric circle with central blood vessels, and inner and outer circumferential lamellae. Therefore, the anatomical structure of the human cortical bone is not the same as that of rodents. However, the inner circumferential lamellae's endosteal bone surface in humans must histologically correspond to the endosteal cortical bone surface in mice and the bone surface of the Haversian canal of osteons. Nevertheless, animal studies still have limitations in reproducing the cortical porosity in humans; thus, further experiments are necessary.

Intermittent PTH administration induced femoral cortical porosity that progressed from the metaphysis to the diaphysis in mice. After PTH administration, endomucin-positive blood vessels and PDGFbb-reactive osteoclasts in ALP/αSMA/VCAM1-reactive osteoblastic cell layers invade the cortical bone through osteocyte-derived molecules, such as DKK1.

Acknowledgment

Competing Interests

The authors declare they have no competing interests.

Funding Statement

Author Contributions

TH designed the study and drafted the manuscript. MA was the first author to write the original draft, analysis of data, and interpretation of data. HH, TY, XL, QY, and MH contributed to immunohistochemistry. HM, HI, HY, TS, and CJ worked on the statistical analyses and critical reading. NA participated in the discussion, editing, and formatting of the manuscript. All the above authors have read and approved the final manuscript.

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Regions of Interest (ROI) in the Cortical Bone

Distal femora

950μm

Zone 1   700μm

Zone 2   700μm

Zone 3   700μm

posterior   anterior

A

Assumed boxed area for quantification

700μm

100μm

B

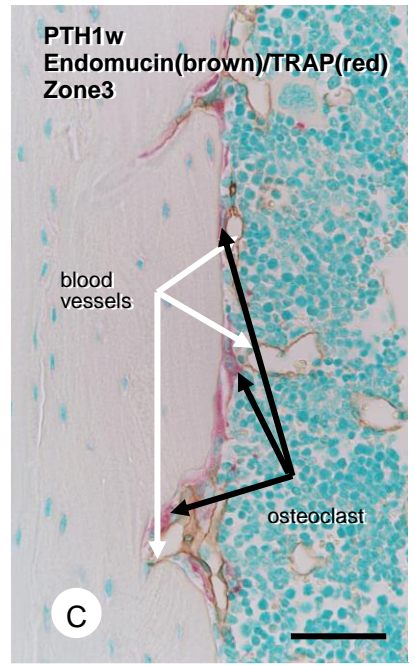**Method for quantifying distance between blood vessels and the endosteal bone surface**

BV

BV

cortical bone

C

A

	control	PTH1d	PTH3d	PTH1w	PTH2w	PTH3w
	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Ⅵ
cortical bone	Ⅶ	Ⅷ	Ⅸ	Ⅹ	Ⅺ	Ⅻ
horizontal	i	ii	iii	iv	v	vi

B

	control	PTH1d	PTH3d	PTH1w	PTH2w	PTH3w
	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Ⅵ
cortical bone	Ⅶ	Ⅷ	Ⅸ	Ⅹ	Ⅺ	Ⅻ

Control1w
Endomucin(brown)/TRAP(red)
Zone1

blood
vessels

A

Control1w
Endomucin(brown)/TRAP(red)
Zone2

blood
vessels

B

Control1w
Endomucin(brown)/TRAP(red)
Zone3

blood
vessels

C

Control1w
Endomucin (brown)/ALP(blue)
Zone1

osteobaslt

blood
vessels

D

Control1w
Endomucin (brown)/ALP(blue)
Zone2

osteoblast

blood
vessels

E

Control1w
Endomucin (brown)/ALP(blue)
Zone3

osteoblast

blood
vessels

F

PTH1w
Endomucin(brown)/TRAP(red)
Zone1

blood
vessels

osteoclast

A

PTH1w
Endomucin(brown)/TRAP(red)
Zone2

blood
vessels

osteoclast

B

PTH1w
Endomucin(brown)/TRAP(red)
Zone3

blood
vessels

osteoclast

C

PTH1w
Endomucin (brown)/ALP (blue)
Zone1

blood
vessels

D

PTH1w
Endomucin (brown)/ALP(blue)
Zone2

blood
vessels
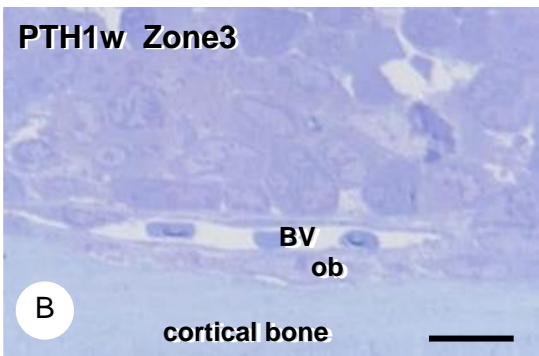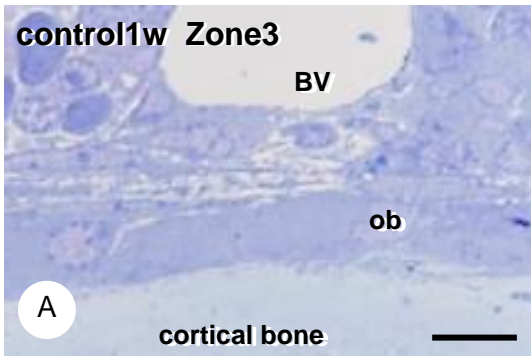
E

PTH1w
Endomucin (brown)/ALP(blue)
Zone3

blood
vessels

F

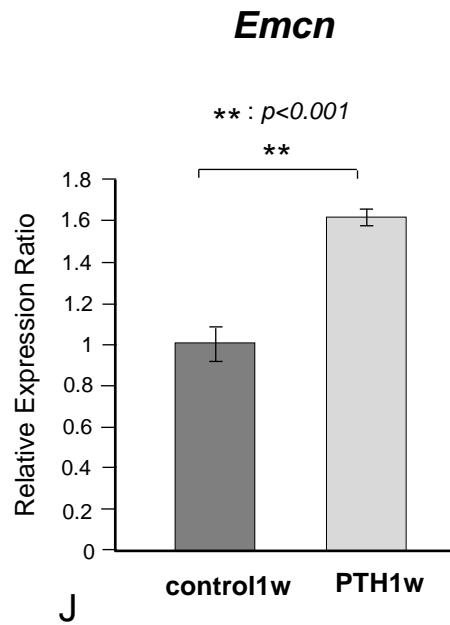# Distance between blood vessels and the endosteal bone surface

Zone1
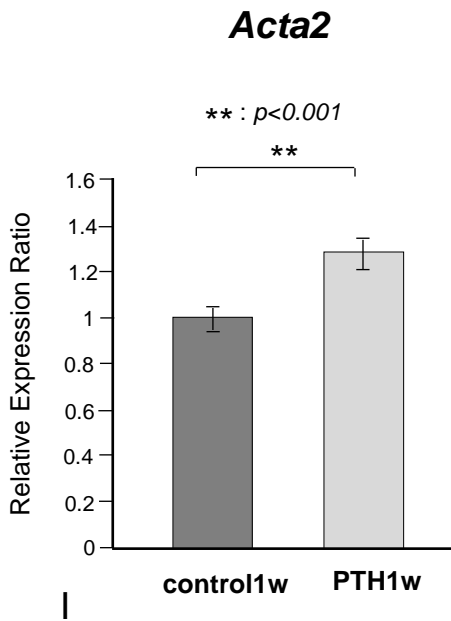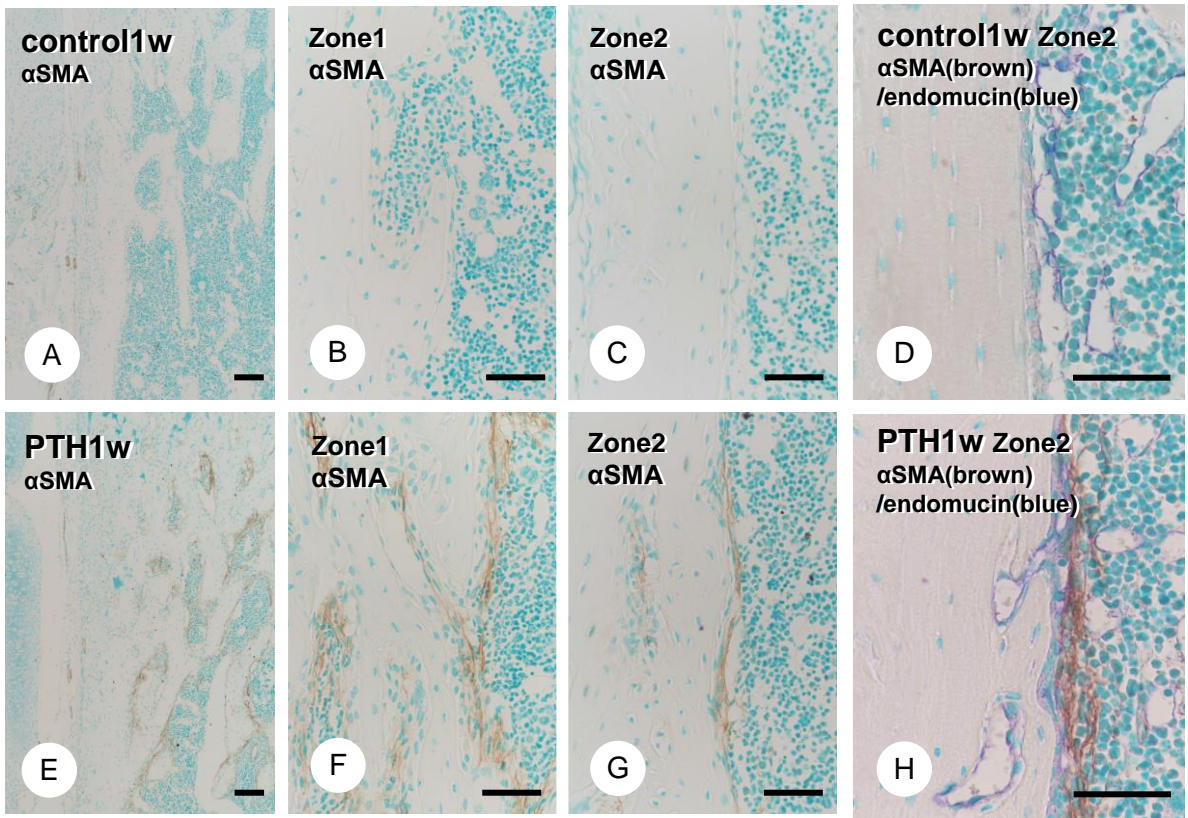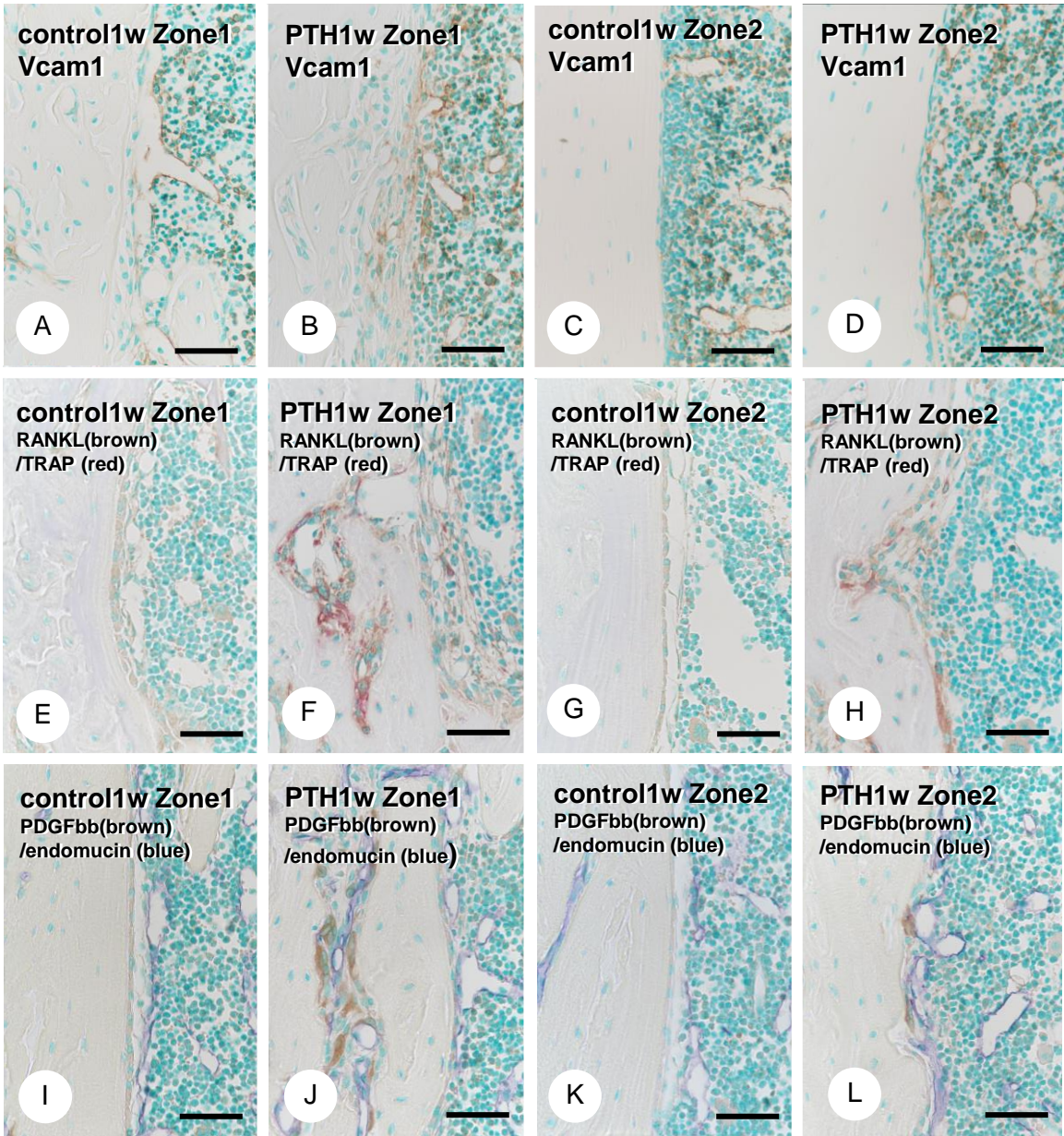
(μm)   ** : *p<0.001*

A   **1day   3days   1week**

Zone2

(μm)   ** : *p<0.001*

B   **1day   3days   1week**

Zone3

(μm)   ** : *p<0.001*

C   **1day   3days   1week**

■ control
□ PTH

Osteoclast numbers

Zone1

(N)   ** : *p<0.001*

D   **1day   3days   1week**

Zone2

(N)   * : *p<0.05*

** : *p<0.001*

E   **1day   3days   1week**

Zone3

(N)   ** : *p<0.001*

F   **1day   3days   1week**

■ control
□ PTH

A — control1w Zone3: BV, ob, cortical bone

B — PTH1w Zone3: BV, ob, cortical bone

C — control1w Zone3: BV, ob, cortical bone

D — PTH1w Zone3: BV, oc, ob, cortical bone

E — PTH1w Zone2: BV, cortical bone

F — PTH1w Zone2 von Kossa: BV, cortical bone

G — PTH1w Zone2: BV, oc, BV, cortical bone

control1w αSMA

Zone1 αSMA

Zone2 αSMA

control1w Zone2 αSMA(brown) /endomucin(blue)

A B C D

PTH1w αSMA

Zone1 αSMA

Zone2 αSMA

PTH1w Zone2 αSMA(brown) /endomucin(blue)

E F G H

Acta2

** : *p<0.001*

**

Relative Expression Ratio

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

control1w   PTH1w

I

Emcn

** : *p<0.001*

**

Relative Expression Ratio

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

control1w   PTH1w

J

Tnfsf11

** : *p<0.001*

Pdgfb

** : *p<0.001*
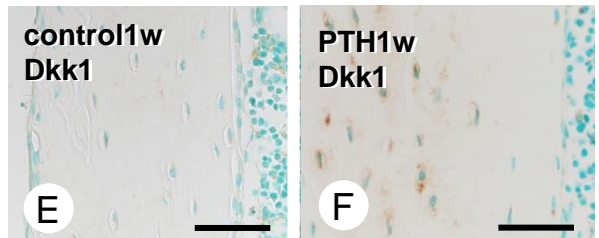
Angiogenesis related gene

Sema3e
Hif1a
Pdgfb
Vash2
Ephb4
Efnb2
Ramp3
Ephb2
Pik3r3
Srpx2
Aplnr
Gas1
Cd276
Dkk1 *
Ckap4*
Plvap*

control1w   **PTH1w**

Osteocyte derived gene

Sost
Mepe
Dmp1
Phex
Tnfsf11
Dkk1

control1w   **PTH1w**

control1w
sclerostin

PTH1w
sclerostin

control1w
Dkk1

PTH1w
Dkk1

control1w
PLVAP

PTH1w
PLVAP

Sost

N.S.

Relative Expression Ratio

control1w   PTH1w

Dkk1

** : *p<0.001*
**

Relative Expression Ratio

control1w   PTH1w

Ckap4

** : *p<0.001*
**

Relative Expression Ratio

control1w   PTH1w

Plvap

* : *p<0.05*
*

Relative Expression Ratio

control1w   PTH1w

Figure Legends

Figure 1

ROI in cortical bone and quantification

ROI images named Zone 1, Zone 2, and Zone 3 on the posterior femoral cortical bones are denoted in blue boxes (A). Panel B displays the assumed boxed areas for measuring the distance between blood vessels and endosteal bone surfaces and osteoclast numbers. Panel C illustrates the quantification method for the distance between blood vessels and the endosteal bone surface. The distance between blood vessels and the endosteal bone surface is calculated by dividing the surrounding area between endomucin-positive blood vessels and the cortical bone surface (diagonal blue-lined area) by the cortical bone surface length (red line).

Figure 2

Micro CT and histological images obtained from the femoral cortical bone in control and hPTH [1-34]-administered mice

(A) Micro-CT images of the femora in control and PTH-administered mice. (B) H-E staining of the femora in control and PTH-administered mice. Figs. VII-XII and i-vi reveal highly magnified cortical bone in the sagittal and horizontal sections. PTH-administered femora chronologically show abundant metaphyseal trabeculae (AII-VI, B II-VI) compared to control mice (AI, BI). High-magnification images of the posterior cortical bone exhibit porous or

trabecular bone-like structures 1 week post-PTH administration. This porosity appears

chronologically from the metaphyseal to the diaphyseal regions (A VII-XII, B VII-XII).

Horizontal micro-CT images show that this porosity originated from the endosteal side and

spread towards the mid-region of the cortical bone (A i-vi).

Bars, A-R: 1 mm, a-f: 500 μm, g-l: 100 μm

Figure 3

Immunolocalization of endomucin-positive blood vessels, TRAP-positive osteoclasts, and ALP-positive osteoblasts in control mice

(A-C) The double detection of endomucin-positive blood vessels (brown) and TRAP-positive

osteoclasts (red) in control mice. (D-F) The double detection of endomucin (brown) and ALP-

reactive osteoblasts (blue). Endomucin-positive blood vessels (brown, white arrows) far from

the cortical surface and TRAP-positive osteoclasts on the cortical bone surface in all zones. In

contrast, ALP-positive mature osteoblasts (blue, black arrowheads) are located on the cortical

bone surface (D-F). Endomucin-positive blood vessels never penetrated the ALP-reactive

osteoblastic layers.

Bars, A-F: 50 μm

Figure 4

Immunolocalization of endomucin-positive blood vessels, TRAP-positive osteoclasts, and ALP-positive osteoblasts in mice 1 week after PTH administration

(A-C) The double detection of endomucin-positive blood vessels (brown) and TRAP-positive osteoclasts (red) in PTH-administered mice after 1 week. (D-F) The double detection of endomucin (brown) and ALP-reactive osteoblasts (blue). In Zone 1, there were many TRAP-positive osteoclasts (red, black arrows, A), endomucin-reactive blood vessels (brown, white arrows, A and D), and a thick ALP-positive osteoblastic layer covering the cortical bone surface (D). In Zones 2 and 3, numerous blood vessels with small diameters were apparent on the cortical surface, partially penetrated in the cortical bone with TRAP-positive osteoclasts (B, C). Unlike Zone 1, ALP-positive osteoblasts in Zones 2 and 3 do not form as thick cell layers (E, F).

Bars, A-F: 50 μm

Figure 5

Distance index between blood vessels and the endosteal bone surface and osteoclast numbers in control and PTH-administered mice

(A-C) The distance between blood vessels and the endosteal cortical bone surface. (D-F) Osteoclast numbers in Zones 1-3. The distance between the blood vessels and the endosteal cortical bone surface significantly decreased in PTH groups compared with the control groups

across all periods and zones (A-C). The number of osteoclasts in PTH groups is higher than that

of the control group across all periods and zones (D-F).

Figure 6

Ultrastructural images of cells around the cortical bone surface in control and PTH-administered mice

Light microscopic images of the cortical surface in Zones 2 and 3 in control (A) and PTH mice

1 week after administration (B, E, F). Panels C, D, and G present the TEM images at the

corresponding areas of panels A, B, and E. 1 week after PTH administration, flattened

osteoblasts and osteoclasts with blood vessels are located on the cortical bone surface in Zone

3 (B, D). In Zone 2 with PTH-administered mice, the invading vascular endothelial cells with

osteoclasts at the tip of blood vessels (G). The bone matrix near the tips of invading blood

vessels was mineralized (F).

BV, blood vessel; ob, osteoblast; oc, osteoclast

Bars, A, B, E, F: 20 μm, C, D, G: 10 μm

Figure 7

Histological comparison of the αSMA-positive osteoblastic cell layer and *Acta2 (αSma)* and *Emcn* gene expression in the control and PTH-administered mice

At a lower magnification of αSMA immunohistochemistry, αSMA-positive cells (brown) covered the bone surface in PTH-administered mice after 1 week (Panel E). Only a few αSMA-positive cells were apparent in the periosteum of control mice (Panel A), but there were no αSMA-positive cells on the surface of the endosteal side (Panels B and C). In the PTH group, αSMA-positive thick cell layers were observed in Zone 1 (F). Thin αSMA-positive cell layers were observed in Zone 2 (G). Double αSMA (brown) and endomucin (blue) staining revealed that endomucin-positive blood vessels invading the cortical bone were covered by the αSMA-positive cell layer in PTH-administered mice (H). The control group had no αSMA-positive cell layer around endomucin-positive blood vessels (D). Real-time PCR analysis consistently demonstrated a significant increase in *αSma (Acta2)* and *Endomucin (Emcn)* expression in PTH-administered mice (I, J).

Bars, A-C, E-G: 100μm, D, H: 50μm

Figure 8

VCAM1, RANKL, PDGFbb distribution and gene expression in control and PTH-administered mice

(A-D) VCAM1 immunodetection (brown) in control (A, C) and PTH-administered mice (B, D). A VCAM1-positive thick osteoblastic layer exists on the cortical bone surface in Zone 1 (B), whereas a thin layer was in Zone 2 (D). (E-H) The double detection of RANKL (brown) and

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TRAP (red) in control (E, G) and PTH-administered mice (F, H). Numerous TRAP-positive osteoclasts were located in the RANKL-positive osteoblastic layers in Zones 1 and 2 (F, H). (I-L) The double detection of PDGFbb (brown) and endomucin (blue) in control (I, K) and PTH-administered mice (J, L). There were no PDGFbb-positive osteoclasts in control mice (I, K). PDGFbb-positive osteoclasts were apparent in the cortical porous with endomucin-positive blood vessels in Zone 1 (J) and endomucin-positive blood vessels on the cortical bone surface in Zone 2 (L) of PTH-administered mice. Real-time PCR consistently demonstrated a significant increase in *Rankl (Tnfsf11)* and *Pdgfb* expression (M, N).

Bars, A-L: 50 μm

Figure 9

Osteocyte-derived angiogenic factor, Dkk1, Ckap4, and Plvap immunolocalization and gene expression in PTH-administered mice

Heatmap analysis by RNA sequencing revealed an elevated expression of numerous angiogenic factors, *Hif1a, Pdgfb, Vash2, Ephb4, Efnb2, Ramp3, Ephb2, Pik3r3, Srpx2, Aplnr, Gas1, Cd276, Dkk1, Ckap4, Plvap* (A), and osteocyte-derived molecules, such as *Dmp1, Phex, Tnfsf11,* and *Dkk1* (B) in PTH-administered mice. Sclerostin-positive osteocytes and their canaliculi were apparent in control and PTH-administered mice (C, D). There was no significant difference in the Sost expression in both mice groups (I). Dkk1 immunohistochemistry demonstrated the

intense immunoreactivity in PTH-administered mice (F) compared with control mice (E). Dkk1

expression was significantly elevated after PTH administration (J). Plvap-positive blood vessels

were located close to the cortical bone surface and invaded the cortical bone in PTH-

administered mice (H) but not in control mice (G). *Ckap4* and *Plvap* gene expression were

significantly elevated (K, L).

Bars, C-H: 50 μm

Table Legends

Table 1

Taqman gene expression assays used in the study.

Table 1

Gene name	Symbol	Identification number of applied biosystem
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	Mm99999915_g1
α-smooth muscle actin (αSMA)	*Acta2*	Mm00725412_s1
Endomucin	*Emcn*	Mm00497495_m1
Receptor activator of NF-κB ligand (RANKL)	*Tnfsf11*	Mm00441906_m1
Platelet-Derived Growth Factor-BB (PDGF-BB)	*Pdgfb*	Mm00440677_m1
Sclerostin	*Sost*	Mm00470479_m1
Dickkopf-1 (DKK1)	*Dkk1*	Mm00438422_m1
Cytoskeleton-associated protein 4 (CKAP4)	*Ckap4*	Mm01236268_m1
Plasmalemma vesicle-associated protein (PLVAP, PV-1)	*Plvap*	Mm00453379_m1