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Constitutively Active PTH/PTHrP Receptor Specifically Expressed in Osteoblasts Enhances Bone Formation Induced by Bone Marrow Ablation

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Abstract

Bone is maintained by continuous bone formation by osteoblasts provided by proliferation and differentiation of osteoprogenitors. Parathyroid hormone (PTH) activates bone formation, but because of the complexity of cells in the osteoblast lineage, how these osteoprogenitors are regulated by PTH in vivo is incompletely understood. To elucidate how signals by PTH in differentiated osteoblasts regulate osteoprogenitors in vivo, we conducted bone marrow ablation using Col1a1-constitutively active PTH/PTHrP receptor (caPPR) transgenic mice. These mice express caPPR specifically in osteoblasts by using 2.3 kb Col1a1 promoter and showed higher trabecular bone volume under steady-state conditions. In contrast, after bone marrow ablation, stromal cells recruited from bone surface extensively proliferated in the marrow cavity in transgenic mice, compared to limited proliferation in wild-type mice. Whereas de novo bone formation was restricted to the ablated area in wild-type mice, the entire marrow cavity, including not only ablated area but also outside the ablated area, was filled with newly formed bone in transgenic mice. Bone mineral density was significantly increased after ablation in transgenic mice. Bone marrow cell culture in osteogenic medium revealed that alkaline phosphatase-positive area was markedly increased in the cells obtained from transgenic mice. Furthermore, mRNA expression of Wnt-signaling molecules such as LRP5, Wnt7b, and Wnt10b were upregulated after marrow ablation in bone marrow cells of transgenic mice. These results indicate that constitutive activation of PTH/PTHrP receptor in differentiated osteoblasts enhances bone marrow ablation-induced recruitment, proliferation, and differentiation of osteoprogenitors.

Adult bone is maintained through continuous bone formation coupled with bone resorption. Various signals caused by systemic hormones and cytokines regulate the activities of osteoblastic cells such as proliferation, differentiation, and matrix synthesis (Harada and Rodan, 2003). Cells in the osteoblastic lineage are heterogeneous hence comprised of immature and mature cells in various stages (Aubin, 1998a,b). Commitment to the osteoblastic lineage from mesenchymal stem cells is determined by the transcriptional factor, Runx2 (Komori et al., 1997), followed by another transcriptional factor Osterix (Osx) (Nakashima et al., 2002). Early mature osteoblasts abundantly express type 1 collagen to synthesize bone matrix. Mature osteoblasts sequentially express markers such as alkaline phosphatase (ALP), osteopontin and osteocalcin, and eventually perform matrix mineralization. Some of the terminally differentiated osteoblasts are either embedded in bone to become osteocytes or become dormant on bone surface as lining cells. Expression of regulatory cytokines and their receptors vary upon the differentiation stages of the developmental sequence (Liu et al., 2003). Therefore effects of hormones on the osteoblast lineage depend on the developmental stages of cells and their microenvironment including recovery phase after bone injury.

Parathyroid hormone (PTH), a principal regulator of calcium homeostasis, plays crucial roles in bone metabolism (Qin et al., 2004). Intermittent administration of PTH leads to accumulation of bone mass and thus it is used clinically as an anabolic agent for osteoporosis. PTH administration, especially in intermittent regimens, activates proliferation and differentiation of osteoblastic cells in vivo. In bone, receptors for PTH (PTH1R, PTH/PTHrP receptor) are expressed solely in osteoblastic cells (Fermor and Skerry, 1995). PTH/PTHrP receptor is ubiquitously expressed among cells in the osteoblastic lineage from very early immature cells to terminally differentiated mature cells (Candelieri et al., 2001). Therefore, PTH action on bone could be the result of modulation of cells in any stage in the osteoblast lineage.

Intracellular cAMP-PKA pathway is a major signaling cascade in the downstream of G protein coupled-PTH/PTHrP receptor, and promotes differentiation of osteoblastic cells by a variety of machineries shown by previous studies (Ishizuya et al., 1997; Qin et al., 2005; Li et al., 2007). Jansen-type mutation (H223R) renders constitutive activation of PTH/PTHrP receptor, in which cAMP is accumulated in a ligand-independent manner (Schipani et al., 1996). The transgenic mice overexpressing H223R under 2.3 kb Col1a1 promoter [Col1a1-constitutively active PTH/PTHrP receptor (caPPR) Tg-mice] resulted in an increase in precursors and mature osteoblasts as well as their functions, leading to a substantial increase in trabecular bone volume (Calvi et al., 2001). Therefore, PTH/PTHrP receptor expressed by type 1 collagen expressing osteoblastic cells plays as a crucial mediator of PTH action in bone.

Recently, stem cell niche has been intensively studied. Hematopoietic stem cells (HSCs) are shown to be maintained in a specific microenvironment comprised of osteoblasts and extracellular matrix molecules, and the signals that target osteoblasts such as PTH have been shown to regulate HSCs in an indirect, niche-mediated manner (Calvi et al., 2003; Stier et al., 2005; Adams et al., 2007). In contrast to HSCs, the regulation of osteoprogenitors by their microenvironment is still elusive.

Bone marrow ablation provides an in vivo system to study tissue regeneration based on a sequence of bone formation and resorption at a well-defined anatomic site in vivo. Morphologic features are correlated with the pattern of regeneration after fracture or bone injury (Suva et al., 1993). Following ablation in bone marrow, blood clot fills the ablated cavity. Then, there is a phase of bone formation and newly formed bone replaces the initial blood clot and fills the ablated region of marrow cavity. In these processes, osteoblastic cells

proliferate first and these were suggestions that certain factors from the blood clot would support such osteoblastic activity.

To examine whether PTH signaling in osteoblasts regulate post-injury regeneration of bone in vivo, we conducted bone marrow ablation in transgenic mice harboring caPPR whose expression is driven by *Col1a1* promoter.

Experimental Procedures

Animals

Col1a1-caPPR transgenic mice in a FVB/N background were previously reported (Calvi et al., 2001). Briefly, a mouse 2.3 kb fragment of *Col1a1* promoter was ligated upstream to the entire coding region of human mutated Jansen type PTH/PTHrP receptor (HKrk-H223R), along with the cloning vector pcDNA1 sequence that contains a poly(A) signal. Male transgenic mice were crossed with female FVB/N mice to generate littermates. Eight-week-old female transgenic mice and their wild-type littermates were used for experiments. Genomic DNA from tail was used for PCR genotyping, using a forward 5'-GAGTCTACATGTCTAGGGTCTA-3' and a reverse 5'-TAGTTGGCCACGTCCTGT-3' primer under following conditions: 94°C for 1 min, 58°C for 45 sec, and 72°C for 1 min for 35 cycles. All experiments were performed according to institutionally approved guidelines for animal welfare.

Bone marrow ablation model

The mice were anesthetized with avertin (2% 2,2,2-tribromoethanol in saline) injected intraperitoneally at the dosage of 15 μ l per gram body weight before the operation. Right femora were operated while left femora were untreated and used as an internal control. After skin incision, cruciate ligaments of the knee were carefully separated using a dental excavator, and a hole was made in the intercondylar region of femora using a 26-gauge needle. The endodontic instruments (K-file, #20, 25, 30, 35) (GC, Tokyo, Japan) were used in a stepwise manner to remove bone marrow tissue. X-ray radiographs were taken to confirm if the direction of file insertion was correct. Mice were sacrificed 3, 7, 14, or 28 days after the operation.

Three-dimensional micro-CT analysis of bone

A micro-CT apparatus (Scan Xmate-E090, Comscan Techno, Tokyo, Japan) was used for imaging. 3D-microCT images were analyzed and quantified using an image analysis software (TRI/3D-BON, Ratoc system engineering, Tokyo, Japan). The distal metaphyseal regions of femora between the growth plate and 2.0 mm proximal to the growth plate were used for analysis.

Measurement of bone mineral density (BMD)

BMD of one-third distal femora was measured by dual X-ray absorptiometry using a PIXImus system (GE Lunar, Madison, WI).

Histological analysis

For histological analysis, femora were fixed with 4% PFA, decalcified in 10% EDTA, and embedded in paraffin in a lengthwise manner. Seven micrometer-thick horizontal serial sections of distal metaphyses were made. To label proliferating stromal cells in vivo, BrdU (50 μ g/g body weight) was injected intraperitoneally 60 h after marrow ablation, and mice were sacrificed 12 h thereafter (Day3). Immunohistochemistry for BrdU-positive stromal

cells was performed using BrdU In-Situ Detection Kit (BD Science, NJ). BrdU-positive stromal cells around bone surfaces (upto 10 μ m from surface) were counted.

Bone marrow cell cultures

For CFU-f ALP (ALP-positive colony-forming unit) assay, bone marrow cells were plated at 3.5×10^4 cells/cm² in 60 mm dish (Costar, Washington, DC) and cultured for 18 days in DMEM containing 10% FBS, 50 μ g/ml ascorbic acid, 10 mM sodium beta-glycerophosphate and 100 nM dexamethasone. ALP staining was performed with BCIP/NBT substrate (Boehringer, Ingelheim, Germany) and cells were counterstained with Nuclear fast red solution (Sigma, MO). A colony comprised of more than 30% of ALP-positive cells was defined as ALP^{high} colony. For ALP-positive cell development, bone marrow cells were plated at a 8.0×10^5 cells/cm² in 12-well dish (Costar) and cultured for 21 days in alpha-MEM containing 10%FBS, 50 μ g/ml ascorbic acid, and 10 mM sodium beta-glycerophosphate. ALP staining was performed with BCIP/NBT substrate.

Quantitative real-time PCR analysis

After 3 days of bone marrow ablation, entire bone marrow tissues from both ablated and control femora were carefully taken out using H-files (endodontic instrument, GC, Japan), and cells were immediately lysed in TRIzol reagent (Invitrogen, CA). Total RNA (500 ng) was treated with DNase I (Invitrogen) prior to reverse-transcription. First-strand cDNA was synthesized using SuperScript II transcriptase and oligo(dT)₁₂₋₁₈ primers (Invitrogen). Quantitative real-time PCR analysis was carried out using iCycler (Bio-rad, CA) and iQ5 data analyzing software. The reaction was performed in a 25 μ l reaction mixture containing 2 μ l cDNA samples, 1 μ l sense and anti-sense primer mix (5 μ M), and 12.5 μ l iQ SYBR Green Supermix (Biorad, CA). The primer sequences were designed based on Beacon Designer (Bio-rad) program, and were listed below. The PCR condition was 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec for 40–50 cycles.

Primer sequences for quantitative real-time PCR

Human mutated PTH1R (caPPR, H223R)			
Forward	5'-tggacctgctgacatagtg-3'	(NM_000316: 2001–2020)	
Reverse	5'-aggctcctcacaagatcctc-3'	(pcDNAI: 2331–2349)	
Product size	115 bp		
Gene	Forward (5'–3')	Reverse (5'–3')	Product size (bp)
Runx2	tggcttgggttcaggttaggg	tcggttcttaggtcttgagtg	106
Osx	gcaactggctaggtggtggtc	gcaaagtcagatgggtaagtaggc	115
LRP5	gctccgctctcagttccc	gacagacagcatcgagtttagc	117
LRP6	aatggtcagtcggttgaaagc	ggtgctggctcctcagttgg	101
Wnt3a	atgacagtcgctcgagatgg	ggtaggttcgcagaagttggg	150
Wnt7b	ctgctgccaacctccacctc	tgctcctgacctctcctgaacc	99
Wnt10b	tctcactacagcccagaacc	gcctccaagagcctgacaag	126

Statistical analysis

The results were expressed as mean values $\pm$ SD. Statistical evaluation was conducted based on Mann–Whitney's *U*-test. A *P*-value of <0.05 was considered significant.

Results

3D-microCT analysis on the cortical trabeculation with normal hematopoietic marrow space in adult Col1a1-caPPR transgenic mice under steady-state conditions

Constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells resulted in an increase in trabeculation in marrow space and porosity in cortical bone as reported previously (Calvi et al., 2001). 3D-microCT cross-sectional images of distal metaphyses of the femora were acquired for stereographic structural analysis (Fig. 1a–e). In transgenic mice, trabecular bone in marrow cavity was increased, and porosity in cortical bone was increased but this was only at specific areas of the cortex, usually at antero-posterior and lateral corners of marrow space (Fig. 1c,d, closed arrowheads). The areas of cortical porosity were always associated with extension of trabeculation into marrow space. These areas of cortical trabeculation were similar to trabecular-bone-like area (TBLA) described in the mutant mice with conditional inactivation of BMPRIA (Zhang et al., 2003), which is claimed to be responsible for the increase in the number of HSCs in these mice. Consistent with previous reports three dimensional (3D), fractional trabecular bone volume (BV/TV) of the 8-week-old transgenic mice was about twofold higher than that of controls (Fig. 1e).

Hematoxylin and eosin (H&E) staining indicated that cellularity in marrow space was similar in the transgenic mice and wild type under steady-state conditions (Fig. 1f,h). Small round hematopoietic cells were dominant in marrow cavity despite increased trabeculation in the transgenic mice (Fig. 1h). This was also observed in wild type (Fig. 1f). Under high magnification images, few layers of lining cells were observed on endosteal surface in wild-type mice and on endosteal surface without cortical trabeculation (Fig. 1g). However, in transgenic mice, stromal cells occupied the intertrabecular marrow space in TBLAs of the endosteum in transgenic mice (Fig. 1i). These data indicate that hematopoietic marrow spaces were basically similar in Col1a1-caPPR transgenic mice and in wild type under steady-state conditions, except in specific areas of the endosteum.

Bone marrow ablation enhanced during constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells recruitment and proliferation of stromal cells in marrow cavity

Bone marrow ablation was conducted in the femoral marrow using dental files. After 3 days of ablation (Day3), recruitment of stromal cells from the trabeculae to the ablated area was observed both in wild-type and transgenic mice (Figs. 1a,b and 2d,e). In wild-type mice, the blood clot was largely remained in the ablated area, and domination of stromal cells was only partial (Fig. 2c). In contrast, stromal cells extensively occupied the ablated area in transgenic mice (Fig. 2f), suggesting that recruitment and/or proliferation of stromal cells could be enhanced in transgenic mice. Importantly, proliferation of inside and outside the cells was also observed in bones outside the ablated area in transgenic mice (Fig. 2d, inside and outside the dotted line). In contrast, it was observed only in area adjacent to the ablated region in wild-type mice (Fig. 2a, dotted line). In transgenic mice, the area of domination by stromal cells was more extensive and stromal cells were also numerous in the endosteal surface that is far from the ablated area (Fig. 2d,e, open arrowheads, TBLAs were showed by closed arrowheads). To evaluate proliferation of stromal cells in marrow cavity, BrdU was injected for mice 12 h before sacrificed on Day3. BrdU-positive stromal cells around bone surfaces were approximately 40% higher in transgenic mice after marrow ablation (Fig. 2h). Therefore, constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells enhanced recruitment and proliferation of stromal cells in marrow cavity especially “outside” the ablated area after bone marrow ablation.

Constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells enhanced de novo bone formation “within” the ablated area

Next, we examined 3D BV/TV of the ablated area based on 3D-microCT analysis (Fig. 3a–h) to evaluate de novo bone formation induced by marrow ablation. After 3 days of ablation (Day3), no newly formed bone in the ablated area was apparent both in wild-type and transgenic mice (Fig. 3a,e). After 7 days of ablation (Day7), reticular bones filled the ablated area in marrow cavity both in wild-type and transgenic mice (Fig. 3b,f). BV/TV of a cylindrical area (0.35 mm in diameter, upto 2 mm from the growth plate) within the ablated area was higher in transgenic mice than in wild-type mice (Fig. 3b vs. f and i), suggesting that stromal cells in the ablated area performed bone formation. Therefore, constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells enhanced de novo bone formation in the ablated area. Although reticular bones in the ablated area underwent gradual resorption after 14 and 28 days regardless of the genotypes, transgenic mice constantly showed significantly higher BV/TV than wild-type mice (Fig. 3d,h–j).

Constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells induced extensive de novo bone formation in marrow cavity “outside” the ablated area and trabeculation of cortical bone after marrow ablation

We further examined areas outside the ablated area within bone marrow by 3D cross-sectional microCT and histological analysis. In wild-type mice, de novo bone formation was restricted to areas adjacent to the ablated area (Fig. 4f, asterisk, dotted circle), and no obvious change in cortical bone was apparent (Fig. 4f, arrowheads). In contrast, extensive de novo bone formation outside the ablated area was observed in transgenic mice, and the entire bone marrow cavity was filled with trabecular bone after 7 days of ablation (Day7) (Fig. 4h, asterisk). Furthermore, an increase in trabeculation within the cortex (Fig. 4h, open arrowheads), and ossification around the ablated area was observed in transgenic mice on Day7 (Fig. 4h, dotted circle). These alterations were further enhanced on Day14 in transgenic mice (Fig. 4l). Trabeculae in marrow cavity after ablation were denser and thicker compared to Day7, and trabeculation was induced further, especially at most periphery (Fig. 4l, arrows). It was also observed that a part of trabeculation of cortical bone extended outside the cortex (Fig. 4l, open arrow), suggesting osteoprogenitor activities i.e., proliferation and differentiation were extensively enhanced in marrow cavity of transgenic mice.

H&E staining on Day7 cross-sectional sections showed that hematopoietic marrow cavity was largely spared in wild-type mice except in the ablated area where newly formed bones and intertrabecular stromal cells existed (Fig. 4n,o). On the other hand, the entire bone marrow cavity was dominated with reticular bone and intertrabecular stromal cells in transgenic mice, and a few cells of hematopoietic marrow were observed (Fig. 4p,q). Furthermore, an integration of trabecular bone in marrow cavity and cortical bone undergoing trabeculation were observed in transgenic mice (Fig. 4p), but not in wild-type mice (Fig. 4n). These data suggest that proliferation and differentiation of osteoprogenitors were significantly enhanced in transgenic mice expressing constitutively active PTH receptor after bone marrow ablation to the extent of obliteration of hematopoietic marrow cavity.

Constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells increased BMD of distal femora after marrow ablation

X-ray radiography of distal femora showed that bone marrow ablation caused significant increase in radio-opacity throughout the metaphysis in transgenic mice (Fig. 5a). We analyzed BMD within one-third distal femora to determine the total levels of mineralization. BMD of both ablated and contralateral sides were measured based on γ DEXA, and BMD of

the ablated side relative to the control was calculated (BMD fold induction). In wild-type mice, this parameter was slightly increased due to newly formed bone in marrow cavity after Day7 and Day14 (Fig. 5b, dotted line). In transgenic mice, this parameter was significantly increased after marrow ablation on these time points (Fig. 5b, solid line). Therefore, the overall effect of bone formation in marrow cavity and simultaneous trabeculation of cortical bone in transgenic mice was an increase in BMD, suggesting that enhanced osteoprogenitor activities at the expense of hematopoiesis in marrow cavity after ablation resulted in extensive mineralization in the presence of constitutively activated PTH/PTHrP receptor in Col1-expressing osteoblastic cells.

Constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells enhanced proliferation of osteoprogenitors ex vivo

To analyze the mechanisms how osteoprogenitors activities were super-enhanced in response to marrow ablation in transgenic mice, we conducted ex vivo culture using bone marrow cells. First, we conducted CFU-f ALP assay (ALP-positive colony-forming unit-fibroblast) to test if the frequency of osteoprogenitors of transgenic mice in bone marrow was enhanced. Bulk bone marrow cells were plated at a clonal density (3.5×10^4 cells/cm²) in 60 mm dish and cultured for 18 days in osteogenic medium in the presence of dexamethasone. A colony comprised of more than 30% of ALP-positive cells was defined as ALP^{high} colony. Although the number of ALP^{high} colony was not significantly different, the number of ALP^{low} colony tended to be higher in transgenic mice (Fig. 6a–d). Therefore, the frequency of osteoprogenitors in bone marrow was unaltered in transgenic mice at least based on this assay. Next, ALP-positive cell development of bone marrow cells was performed to see if proliferation of osteoprogenitors of transgenic mice was enhanced ex vivo. Bulk bone marrow cells were plated at a relatively high density (8.0×10^5 cells/cm²) in 12-well dish to allow cellular interactions, and cultured for 21 days in osteogenic medium. Bone marrow cells of transgenic mice gave rise to ALP-positive cells that dominated larger areas of culture dish, and ALP-positive cell area was about 2.5-fold higher in transgenic mice than in wild-type mice (Fig. 6e,f). Therefore, proliferation/differentiation of osteoprogenitors of transgenic mice was enhanced. These observations are consistent with the previous report (Kuznetsov et al., 2004). Therefore, constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblasts enhanced proliferation of osteoprogenitors ex vivo, but did not alter the frequency of clonal osteoprogenitors in vivo.

Constitutive activation of PTH/PTHrP receptor in Col1-expressing osteoblastic cells modulated mRNA expression of Wnt-signaling molecules in bone marrow cells after marrow ablation

In order to elucidate the molecular basis of enhanced osteoprogenitor proliferation in marrow cavity of transgenic mice after ablation (Day3), we conducted quantitative real-time PCR analysis of bone marrow cells after ablation. After 3 days of bone marrow ablation, entire bone marrow tissues including the endosteum from both ablated and control femora were carefully taken out, and cells were immediately lysed for RNA isolation. mRNA expressions of cells from both ablated and control sides were determined, and gene expressions of the ablated side relative to the control were calculated (fold induction of mRNA expression). Mutant PTH/PTHrP receptor (H223R) was expressed in bone marrow cells of transgenic mice in steady-state conditions, and bone marrow ablation enhanced its mRNA expression (Fig. 7a). Next, we examined transcriptional factors essential for osteoblastic differentiation. mRNA expression of Runx2 was reduced <50% in wild-type mice after marrow ablation, while it was maintained in transgenic mice after ablation (Fig. 7b), suggesting that a greater number of stromal cells were committed to osteoblast-lineage in transgenic mice. Osx mRNA expression was similarly induced by marrow ablation in both genotypes (Fig. 7c). Proliferation and differentiation of osteoprogenitors are regulated

by a number of signals. Of these, recent studies report Wnt signaling as regulators of bone mass (Glass and Karsenty, 2007). LRP5 is a putative Wnt coreceptor expressed in cells of the osteoblast lineage (Kato et al., 2002), whose loss- and gain-of-function lead to low and high bone mass, respectively (Boyden et al., 2002; Kato et al., 2002) *in vivo* but not *in vitro*. mRNA expression of LRP5 was induced over threefold in transgenic mice after marrow ablation, while no induction was observed in wild-type mice (Fig. 7d). LRP6 expression was not significantly altered after marrow ablation in both genotypes (Fig. 7e). mRNA expression of Wnt3a, a canonical Wnt, was induced about sixfold in wild-type mice, but not in transgenic mice after ablation (Fig. 7f). Wnt7b is expressed by osteoblastic cells and required for osteoblastic differentiation (Li et al., 2005). Wnt7b mRNA expression was induced about fourfold in transgenic mice after ablation, but not in wild-type mice (Fig. 7g). Wnt10b inhibits adipocyte differentiation and enhances osteoblast differentiation of mesenchymal stem cell (Bennett et al., 2007). Wnt10b mRNA expression was induced about fourfold in transgenic mice after ablation, but not in wild-type mice though statistically not significant (Fig. 7h). Overall, these data suggest that enhanced proliferation of osteoprogenitors in marrow cavity of transgenic mice after ablation might be associated with altered expression of Wnt-signaling molecules.

Discussion

Our data indicate that expression of caPPR in type 1 collagen expressing osteoblastic cells enhanced proliferation and differentiation of osteoprogenitors in response to bone marrow ablation. Our model of bone marrow ablation established a defined cylindrical area to be removed from marrow cavity, and cellular modulation started from the blood clot recruit cell populations in marrow cavity including undifferentiated mesenchymal stem cells of a quiescent cell population and committed osteoprogenitors of a transient amplifying cell population. Importantly, in wild type, bone marrow cells outside the ablated area did not respond to bone marrow ablation. In contrast, in ca PPR-tg mice, those cells located outside the ablated area strongly responded and changed their shape on day 3 and formed new bone on day 14 and on day 28. These cells rapidly proliferate and differentiate into osteoblastic cells to build up new bones.

It is known that only osteoblastic cells, but not other cell lineages like chondrocytes and adipocytes, are formed in the ablated area of marrow cavity, although the mechanisms underlying this phenomenon is still to be understood. According to our histological findings, these stem/progenitor cells were present in the area between ablated area and endosteal surface (inner surface of the cortex) in transgenic mice. Only low number of these stem/progenitor cells was recruited from trabecular bones adjacent to the ablated area in wild-type mice.

Interestingly, there are areas of trabeculation of cortical bone in transgenic mice that closely resemble TBLA of BMPRIA mutant mice (19), which was previously claimed to be the site of HSC niche on the endosteum. Although whether these stem/progenitor cells were recruited from specific TBLAs in the cortex could not be determined, complete trabeculation of cortical bone was observed after 7 days of ablation in transgenic mice, suggesting that these TBLA would have developed after marrow ablation. This finding may support the notion that osteoblastic stem/progenitor cells that are persistent at specific sites on the endosteal surface in transgenic mice are able to respond to regional stimuli, amplify themselves swiftly and cause super-enhancement of bone formation in marrow cavity. Therefore, in addition to previously known actions of PTH *i.e.*, activation of proliferation and differentiation of osteoblastic cells, PTH signal interacting with injury stimuli such as bone marrow ablation in relatively mature osteoblastic cells might play significant roles in supporting osteoblastic stem/progenitor cell populations. It is intriguing that mature

osteoblasts provide niche for HSCs, and PTH signal activates niche function of osteoblasts (16). This interaction between caPPR signal and bone marrow ablation to enhance new bone formation in the area outside the ablated area is associated with an increase in LPP5 Wnt 10. Whether they also provide niche for mesenchymal stem cells and how PTH signal modulates are the subject of further studies.

Abbreviations

PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
PPR	PTH/PTHrP receptor
caPPR	constitutively active PPR
Col1	type 1 collagen
cAMP	cyclic adenosine monophosphate
BV/TV	bone volume per tissue volume
LRP5	low density lipoprotein receptor-related protein 5

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Fig. 1.

Three-dimensional analysis of bone in Coll1a1-caPPR transgenic mice. Cross-sectional 3D-microCT images of the distal metaphyses of the femora of Coll1a1-caPPR transgenic mice (Tg) and wild-type mice (Wt), at 2 mm (c,a, 2 mm, left panel) and 1 mm (d,b, 1 mm, right panel) proximal to the growth plate, respectively (closed arrowheads: sites of trabeculation in cortical bone in transgenic mice). Bone volume/tissue volume (BV/TV, %) of 2 mm trabecular regions proximal to the growth plate (e) (* $P < 0.05$, $n = 6$ per group). Coll1a1-caPPR transgenic mice (Tg) showed higher trabecular BV/TV and cortical trabeculation. H&E staining, 40 $\times$, gross view (f,h) and 200 $\times$, endosteal regions (g,i) (Scale bar: c: 500 μ m, d: 100 μ m). Coll1a1-caPPR Tg mice showed normal hematopoietic marrow space, but stromal cells occupied the intertrabecular marrow space in TBLAs of the endosteum.

Fig. 2.

Col1a1-caPPR enhanced early recruitment and proliferation of stromal cells in marrow cavity after bone marrow ablation. H&E staining of wild-type (a–c) and *Col1a1-caPPR* transgenic mice (d–f), 3 days after ablation (Day3). Left panel: 40×, gross view (closed arrowheads: sites of cortical trabeculation, open arrowheads: stromal cells recruited from distant endosteal surfaces), right upper panel: 200×, stromal cells emanating from trabeculae (arrows: the direction to the ablated area) and right lower panel: 200×, ablated areas in marrow cavity. (Scale bar: left: 500 μm, right: 100 μm) The number of BrdU-positive stromal cells around bone surfaces 3 days after ablation (Day3) (g,h). BrdU was injected 12 h before sacrificed. *Col1a1-caPPR* transgenic mice enhanced recruitment and proliferation of stromal cells in marrow cavity after bone marrow ablation.

Fig. 3. Coll1a1-caPPR enhanced de novo bone formation within the ablated area. Saggital 3D-microCT images of the distal metaphyses of the femora (a–h), after 3, 7, 14, and 28 days after marrow ablation (Day3, 7, 14, and 28, respectively). (top: ablated area only, bottom all area) Bone volume/tissue volume (BV/TV, %) of the ablated area (2 mm cylindrical area proximal to the growth plate) (i,j) (* $P < 0.05$, ** $P < 0.01$, $n = 6$ per each group, Wt: dotted line, Tg: solid line). Coll1a1-caPPR transgenic mice constantly showed higher BV/TV in the ablated area, despite gradual resorption.

Fig. 4.

Colla1-caPPR induced extensive de novo formation in marrow cavity outside the ablated area and trabeculation of cortical bone after marrow ablation. Cross-sectional 3D-microCT images of the distal metaphyses of the femora after 3, 7, and 14 days after marrow ablation (Day3, 7, 14, and 28, respectively) (a–l). The ablated area was depicted by dotted circles. Domination of marrow cavity by newly-formed bone (f–k, asterisk). Induced trabeculation in Tg (h, open arrowheads). Intact cortical bone in Wt (f, closed arrowheads) on Day7. Significant trabeculation induced at most periphery in Tg (l, arrows) and extended trabeculation outside the cortex (l, open arrow). Bone volume/tissue volume (BV/TV, %) of the entire trabecular regions (2 mm area proximal to the growth plate) was shown below. H&E staining on Day7 (m) (Wt:left panel, Tg: right panel, scalebar: 500 μ m). Residual hematopoietic marrow in Tg after marrow ablation (n,o). The entire marrow cavity was subjected to de novo bone formation after marrow ablation in Tg (p,q).

Fig. 5.

Colla1-caPPR induced an increase in BMD of the distal femora after marrow ablation. X-ray radiographs of left (control) or ablated (right) femora in Wt or Tg mice on Day7 (a). The ablated area was depicted by dotted lines. BMD of the ablated femora relative to the control on Day3, 7, and 14 (b, Wt: dotted line, Tg: normal line). Relative BMD in the ablated femora was significantly increased in Tg.

Fig. 6.

Col1a1-caPPR enhanced proliferation of osteoprogenitors ex vivo, but did not alter their frequency in vivo. CFU-f ALP (ALP-positive colony-forming unit-fibroblast) assay (a) of bone marrow cells. Bulk bone marrow cells were plated at a clonal density (3.5×10^4 cells/cm²) in 60-mm dishes and cultured for 18 days in osteogenic medium in the presence of dexamethasone. ALP^{high} colony (comprised of more than 30% of ALP-positive cells) and ALP^{low} colony count per dish [(b) and (c), respectively], and the percentage of ALP^{high} colony (d). The frequency of ALP^{high} colony was not significantly altered in bone marrow cells of Tg. ALP-positive cell development (e) of bone marrow cells. Bulk bone marrow cells were plated at a relatively high density (8.0×10^5 cells/cm²) in 12-well dish and cultured for 21 days in osteogenic medium. ALP-positive cell area per total area in 12-well dish (f). ALP-positive cell area was 2.5-fold higher in Tg.

Fig. 7.

Col1a1-caPPR modulated mRNA expression of Wnt-signaling molecules in bone marrow cells after marrow ablation. mRNA expression levels of human mutant PPR (caPPR, H223R) (a), Runx2 (b), Osx (c), LRP5 (d), LRP6 (e), Wnt3a (f), Wnt7b (g), and Wnt10b (h) of bone marrow cells on Day3 after ablation were quantified by real-time PCR analysis, using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as a reference. mRNA expression levels of cells from the ablated side were normalized to those of the control (contralateral) side, and each data was presented as mRNA fold induction (* $P < 0.05$; ** $P < 0.01$, $n = 4$ per group). caPPR mRNA was expressed only in Tg cells, and its expression was enhanced by marrow ablation.