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Per-1 Is a Specific Clock Gene Regulated by Parathyroid Hormone (PTH) Signaling in Osteoblasts and Is Functional for the Transcriptional Events Induced by PTH

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ABSTRACT

Per-1 is one of the clock genes and is known to regulate various biological events including bone mass determination. Parathyroid hormone is anabolic to bone while the mechanism of its action is not fully understood. Here, we examined the role of PTH on Per-1 gene expression under osteoblast specific PTH signaling. Constitutively active PTH receptor (caPPR) expressed specifically in osteoblasts in transgenic mice activates Per-1 gene expression in bone. This is specific as expression of other clock gene Bmal-1 is not affected by caPPR over-expression. Per-1 is also expressed in osteoblastic cell line. Interestingly, Per-1 expression is required for PTH signaling-induced CRE dependent transcription. This is forming a positive feed back loop in the anabolic action of PTH signaling and Per-1 in bone. These data indicate that PTH singling in osteoblasts activates Per-1 gene expression in vivo in association with its anabolic action in bone at least in part through the regulation of transcriptional events. J. Cell. Biochem. 112: 433–438, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: PARATHYROID HORMONE; OSTEOBLAST; PER-1

Although circadian rhythm has been known to regulate bone metabolism, the molecular detail has not been fully understood [Hart and Eastell, 1999; Bjarnason and Jordan, 2000; Giudice et al., 2010; Kawai and Rosen, 2010]. When bone is labeled by calcein, it is known that bone formation is dependent on a certain circadian rhythm. However, such rhythm could be found in many

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tissues and specific gene related to the circadian rhythm and its function in bone are still to be elucidated [de Crombrugghe, 2005; Schmitt et al., 2005; Fu et al., 2006].

Circadian rhythm is controlled by clock genes. Per encodes period circadian protein and Bmal-1 encodes brain and muscle arnt-like 1(arntl) protein. Clock is encoding kapu. In addition to these, there are more family members in clock proteins such as *CRY* that encodes cryptochrome. Interaction of these genes would be important in determination of cell and tissue specific or general regulation of metabolism and activities. Heterodimer complex containing Clock and Bmal activates Per gene by binding to the E-boxes located in the promoter regions [Gallego and virshup, 2007].

Bone metabolism is under the control of hormones and cytokines and the secretion as well as the actions of these regulators could modulate or would be influenced by circadian rhythm [Hinoi et al., 2006; Trivedi et al., 2010; Wu et al., 2010]. PTH is one of the most important anabolic agents for osteoporosis while the mechanism of its action in bone is not fully understood [Silver and Bushinsky, 2004; Borba and Mañas, 2010; Bukata and Puzas, 2010; Canalis, 2010; de Paula and Rosen, 2010; Gallagher and Sai, 2010]. Here, we examined the relationship between PTH and Per-1 in osteoblasts and bone.

MATERIAL AND METHODS

ANIMALS

Col1a1-caPPR transgenic mice. Col1a1-constitutively active PTH reception (caPPR) transgenic (PPR-tg) mice in FVB/N background were previously described. For Col1a1-caPPR transgenic mice, a 2.3 kb fragment of the mouse Col1a1 promoter was ligated upstream to the entire coding region of human mutated Jansen type PTH/PTHrP receptor (HKrk-H223R). These were inserted into vector pcDNAI sequence that contains poly(A) signal [Calvi et al., 2001]. All experiments were performed according to institutionally approved guidelines for animal welfare.

Measurement of BMD and μCT analysis of bone. Bone mineral density (BMD) was measured based on dual-energy X-ray absorptiometry (DEXA) as described previously. Briefly, BMD (g/cm^2) of the femora was subjected to dual X-ray absorptiometry using a device specifically designed for small animals (PIXI;GE Luner, Madison, WI) [Ono et al., 2008].

Three-dimensional micro-CT analysis of bone. Imaging of distal metaphyses of the femora was performed using a micro-CT (μCT) apparatus (Scan Xmate-E090, Comscan Techno Co., Japan). 3D-μCT images were analyzed and quantified using an automated image analyzer (TRI/3D-BON, Ratoc System Engineering Co., Japan) [Morinobu et al., 2005; Kondo et al., 2005]. Trabecular bone mass was examined in an area with its closest and furthest edges at 0.25 and 0.75 mm, respectively, from the growth plate in the distal ends of the femora.

Cell culture. Mouse osteoblastic MC3T3E1 cells were cultured in α-MEM supplemented with 10% fetal bovine serum. In some cultures, isoproterenol or vehele was added to the medium.

Transfection and luciferase assay. MC3T3-E1 osteoblastic cells were plated on 24-well plates at a density of 10^4 cells per well. Transfection of small interfering RNA (siRNA) and plasmid DNA was performed on day 1 using Lipofectamine 2000 (Invitrogen, US). siRNA for Per-1 were manufactured by Invitrogen (Per-1 Stealth Select RNAi^{TM} siRNA (MSS207533). Stealth^{TM} RNAi Negative Control Low GC Duplex was used as a control. Plasmid DNA containing human mutated PTH/PTHrP receptor (H223R) was previously described. pcDNA3.1 (Invitrogen) was used as a control. For reporter plasmids, pCRE-luc (PathDetect, Stratagene, US) reporter plasmid was used to measure cyclic AMP (cAMP)-response element activity. Renilla luciferase reporter vector pGL4 (Promega, US) was used as an internal control. For transfection, each well received 0.2 μg H223R plasmid DNA or control, 20 pmol siRNA for Per-1 or control, 0.2 μg pCRE-luc and 20 μg Renilla luciferase plasmid combined with 1.0 μl Lipofectamine 2000. The luciferase assay was performed using the Dual-luciferase Reporter Assay System (Promega) 24 h after transfection. Luciferase activity was measured using a luminometer (Lumat LB 9507, Berthold technologies) [Ono et al., 2007].

Quantitative real-time PCR analysis. Humerus from mice or cultured calvariae were frozen immediately after excision, crushed, and homogenized in Trizol reagent (Invitrogen) using a rotary homogenizer (Polytron 3100, Kinematica, Germany). One milligram of total RNA was treated with DNase I (Invitrogen) prior to reverse transcription. First-strand cDNA was synthesized using SuperScript 3 transcriptase and oligo (dT) primers (Invitrogen). Quantitative real-time PCR analysis was carried out using iCycler (Bio-Rad) and iQ5 data analyzing software. The reaction was performed in a 25 μl reaction mixture containing 2 μl of cDNA samples, 1 μl of sense and antisense primer mixture (5 μM), and 12.5 μl of iQ SYBR Green Supermix. The primer sequences were designed based on the Beacon Designer (Bio-Rad) program and are listed in Table I. The PCR conditions were 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles [Mizoguchi et al., 2010].

Statistical evaluation. Data were expressed as mean $\pm$ SD for all values. Statistical significance of the differences was evaluated based on Students' *t*-test.

RESULTS

As for a possible link that is involved in the effects of PTH signaling in bone, we examined target molecules. Since circadian rhythm was reported to associate with bone metabolism, expression levels of clock genes were evaluated. caPPR which was specifically expressed in osteoblasts, increased the steady state levels of Per-1 mRNA expression in bone in vivo about fourfold (Fig. 1A, lane 1 vs. 2). This indicates for the first time that caPPR signaling in osteoblasts activates steady state Per-1 gene expression in bone.

To test whether the effects of caPPR-regulation would be also seen in case of other clock gene, Bmal-1 was examined. However, osteoblast-specific expression of caPPR did not affect Bmal-1 gene expression at all (Fig. 1B, lane 1 vs. 2) in bone. Thus, Per-1 is a specific target of caPPR signaling in osteoblasts in vivo.

PTH activates bone formation in part through its action on osteoblastic niche. Forced expression of caPPR was reported to specifically enhance hematopoietic niche in bone. This was described to be through activation of osteoblasts by acting on

TABLE I. Primer Sequences for Quantitative Real-Time PCR

Gene	Forward	Reverse
Per-1	5′-ggaggctgtaggcaatggag-3′	5′-tctggcaatggcaaggactc-3′
CXCL12	5′-cgccaaggtcgtcgccg-3′	5′-ttggctctggcgatgtggc-3′
Bmal	5′-gcctttcctcttgcgattgc-3′	5′-tcctcaaccatcagcgacttc-3′

CXCL12. Therefore, we examined CXCL12 expression. caPPR signaling specifically expressed in osteoblasts did not significantly alter the levels of CXCL12 expression (Fig. 1C). Thus, Per-1 is a specific target of the action of caPPR in osteoblasts.

To correlate such caPPR action on Per-1 gene in bone and the levels of bone mass, we examined the levels of BMD in bone where Per-1 expression was evaluated. caPPR expression enhanced whole body BMD levels in mice where enhancement of Per-1 gene expression levels was observed (Fig. 2A). caPPR expression also enhanced BMD levels in long bones from which RNA was prepared to examine Per-1 expression (Fig. 2B, whole Femur). The BMD in the midshaft of the femur (Fig. 2C) or tibia also indicated an increase (Fig. 2D, Tibia). μCT quantification indicated caPPR increased BV/TV (Fig. 2E), trabecular thickness (Fig. 2F), and trabecular number

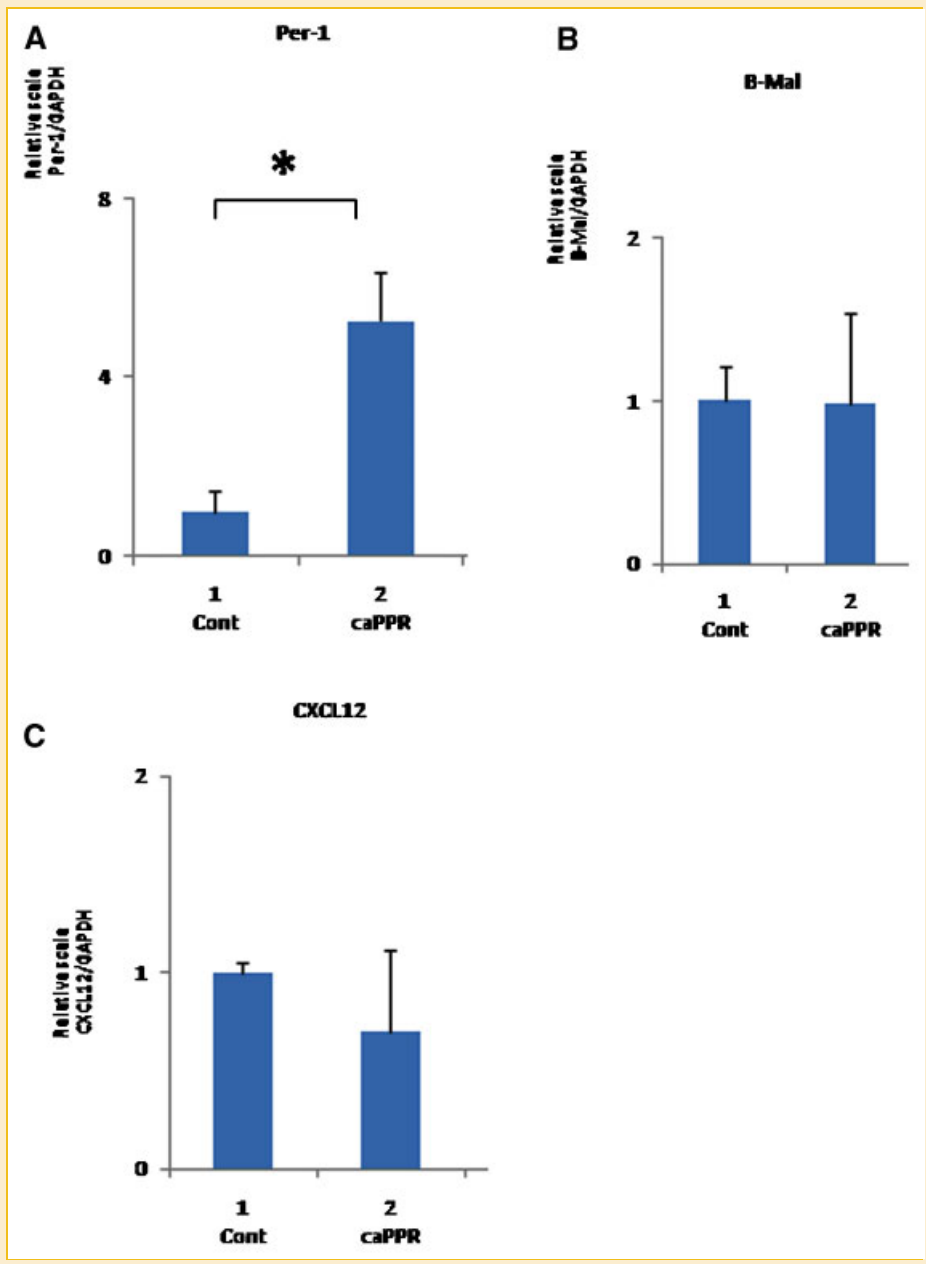

Fig. 1. Per-1 gene expression is enhanced by PTH signaling in bone. (A) Per-1 mRNA expression levels in bone were enhanced by osteoblast-specific expression of constitutively active PTH receptor. (B) Bmal-1 and (C) CXCL12 mRNA expression levels in bone were not enhanced by osteoblast-specific expression of constitutively active PTH receptor. RNA was prepared from long bone of the caPPR transgenic mice or control wild type mice. Quantitative PCR was conducted as described in Materials and Methods Section. Student *t*-test, $^{*}P < 0.05$.

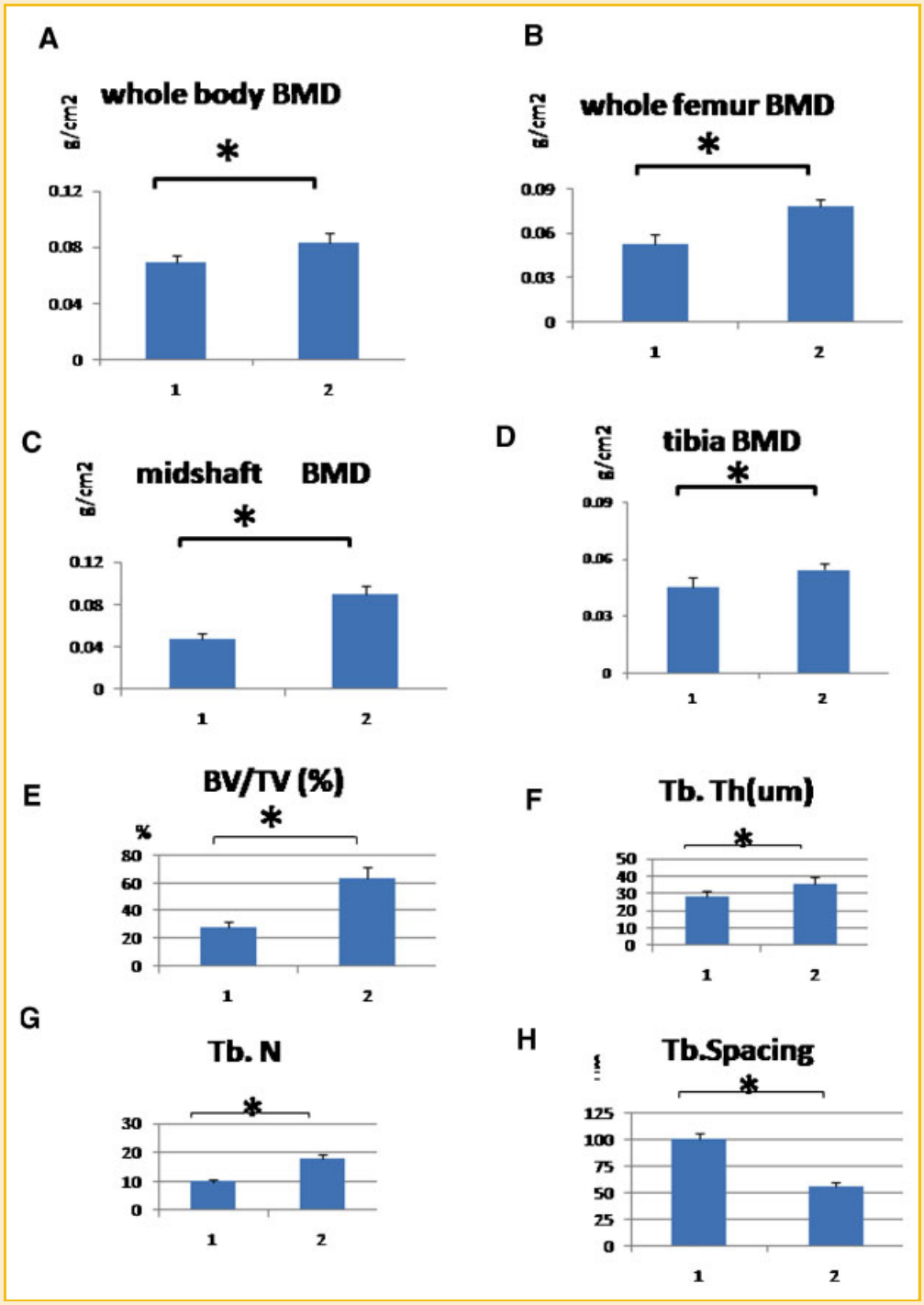

Fig. 2. BMD and μCT parameters are increased due to PTH signaling that enhanced Per-1 expression. BMD (A–E) and μCT analyses (F–H) were conducted in caPPR transgenic (2) mice or control wild type mice (1). Date acquisition was conducted as described in Materials and Methods Section. Student *t*-test,* $P < 0.05$.

(Fig. 2G). Trabecular spacing was reduced in caPPR mice (Fig. 2H). Thus, Per-1 enhancement by caPPR in vivo correlated with the changes in morphological parameters.

As bone is a heterogenous tissue, containing not only osteoblasts but also vascular cells and hematopoietic cells, we tested whether Per-1 is expressed in osteoblasts by using an osteoblastic cell line. Per-1 gene is expressed in osteoblastic MC3T3E1 cells (Fig. 3A). Since PTH accumulates cAMP and activates adenylate cyclase that is also activated by isoproterenol, we examined the effects of isoproterenol and found that Per-1 expression was enhanced in MC3T3E1 cells (Fig. 3A). To see whether such effects are specific to Per-1, we examined Bmal-1. Bmal-1 is expressed in osteoblasts

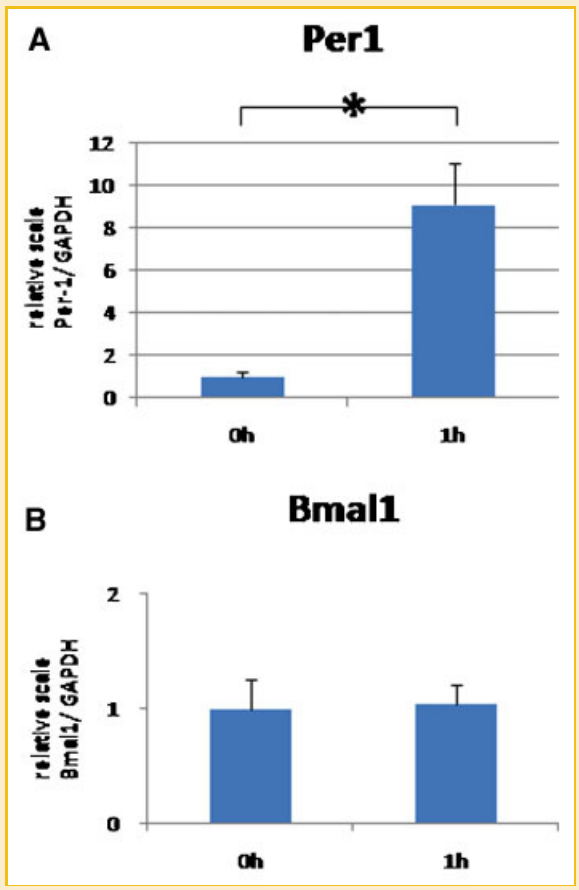

Fig. 3. Per-1 gene is expressed in MC3T3E1 osteoblasts. Per-1 mRNA expression levels in osteoblasts were examined in MC3T3E1 cells. RNAs were prepared from the cells. These cells were untreated (0h) or treated with isoproterenol(1h). Quantitative PCR was conducted as described in Materials and Methods Section. Student *t*-test:$^{*}P < 0.05$.

(Fig. 3B). However, in contrast to Per-1, Bmal-1 levels in osteoblasts were not altered by isoproterenol. Thus, Per-1 regulation by cAMP-related signal in osteoblasts is specific to this gene.

As we found that caPPR specifically expressed in osteoblasts in vivo enhances steady state levels of expression of Per-1 in bone, we further examined whether this expression of Per-1 gene enhanced by caPPR is required for caPPR-induced CRE (cyclic AMP response element) dependent transcription. To do this, endogenous Per-1 expression levels were knocked down by siRNA. The siRNA construct reduced the basal levels of Per-1 gene expression (Fig. 4A). As shown before, overexpression of caPPR in osteoblasts in culture enhanced CRE dependent luciferase expression in the presence of control siRNA (Fig. 4B, lane 1 vs. 2). Under this condition, caPPR-induced enhancement of luciferase activity through CRE was suppressed by the siRNA-induced knockdown of Per-1 mRNA levels (Fig. 4B, lane 2 vs. 4). These data indicated that caPPR enhances Per-1 and in turn Per-1 per se is also required for enhancement of the CRE dependent transcription activated by caPPR.

DISCUSSION

Cell cycle proteins are regulated by clock genes and as PTH regulates cell cycle, it would be affecting clock genes. We identified that caPPR specifically expressed in osteoblasts enhanced steady-state level of Per-1 mRNA in bone in vivo. This sustained Per-1 gene activation by caPPR signaling is specific for Per-1 as caPPR signaling did not alter Bmal-1 gene expression. Importantly, not only Bmal-1 but also other non-clock gene such as CXCL12 is not affected in bone indicating the importance of Per-1 in PTH signaling.

PTH has been shown to activate osteoblastic proliferation through several pathways including molecules such as cyclinD1. We show here that caPPR signaling in osteoblasts enhanced Per-1 expression and it could lead to regulation of cell cycle molecules. Thus, Per-1

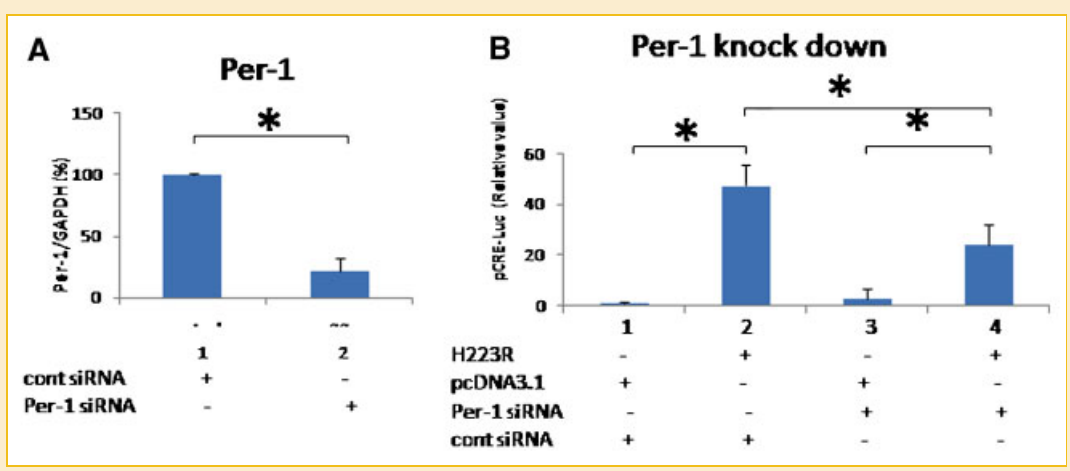

Fig. 4. Per-1 expression is required for PTH-signaling-induced CRE dependent trasncription. (A) Per-1 mRNA levels were significantly reduced in MC3T3-E1 cells treated with Per-1 siRNA compared to the cells treated with control siRNA. (B) Per-1 siRNA suppressed the enhancement in luciferase activity induced by forced expression of the constitutively active H223R mutant form of PTH receptor (caPPR). Data are expressed as means ± SD. Student *t*-test:$^{*}P < 0.05$.

may be one of the critical PTH targets in conjunction with its anabolic action in bone.

Furthermore, Per-1 knock-down by siRNA in osteoblasts suppressed caPPR-induced activation of CRE-dependent transcription. These data indicate that Per-1 plays a critical role in terms of activation of CRE-induced transcriptional activation due to PTH signaling. We speculate that PTH would activate Per-1 and form a positive feedback or positively amplifying system to exert its effects in bone.

In conclusion, we found that Per-1 is a specific target of PTH signaling in bone and modulation of such gene may be beneficial to further enhance PTH action as an anabolic agent for osteoporosis.

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