Identification of a Novel Regulatory Mechanism underlying PTH Anabolic Action on Bone Mass and Injury Repair via Induction of Tob Required for RANKL Expression

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Introduction: PTH is currently used as an only anabolic treatment for osteoporosis. However, the indication for the PTH treatment is limited to severe cases of osteoporosis and the use is also limited up to two years due to the concern for osteosarcoma observed in animal studies. This limitation in the period is hampering the treatment of osteoporosis that requires more than several years of care. In order to overcome these issues, the mechanisms underlying the anabolic actions of PTH would help contemplating to develop non-peptide novel chemical drugs and/or to consider a safer and long term use as an anabolic measure for osteoporosis. However, the mechanism of anabolic actions of PTH on bone is still incompletely understood.

In biological system, homeostasis is often maintained by the balance between the opposite signals. For the anabolic action of PTH on bone, such balancing mechanism is not fully known. We have reported that PTH anabolic actions are modulated by the presence of molecules expressed in osteoblasts (PNAS 2011, PNAS 2012). Tob (Transducer of ErbB2) is expressed in osteoblasts and a member of anti-proliferative BTG-TOB family that interacts with deadenylation complex, CCR4-NOT. This complex includes Cnot3 and we have observed that it regulates bone mass levels (PNAS 2014). We have also shown that Tob is anti-anabolic to bone (Cell 2000, PNAS 2004). However, the relationship between Tob and PTH anabolic actions is not known. We therefore examined the role of Tob in PTH anabolic action.

Methods: Wild type or Tob knockout mice, siRNA for Tob or control siRNA, primary cultures of calvarial cells or osteoblastic cell line, MC3T3E1 were used. RNAs from bone or cells were subjected to quantitative RT-PCR. µCT analyses, histochemical analyses, Western blot analyses and Luciferase assay were carried out.

Results: We first examined the expression of Tob in the bone of mice after intermittent injection with PTH(iPTH). iPTH increased the steady state levels of Tob mRNA expression in bone in vivo and this effect was observed up to the end of the seven day treatment(Fig 1a). As bone contains heterogeneous cell populations, to see if the PTH activates Tob expression in osteoblasts directly, primary cultures of calvarial osteoblasts were used. PTH enhanced Tob mRNA expression in these cells(Fig 1b). PTH exposure increased Tob within 1 hour in these cells(Fig 1c). In vivo, PTH also increased Tob mRNA in bone within 1 hour for a short time course(Fig 1d). This in vivo PTH enhancement of Tob mRNA expression was dose dependent to have highest effects at 80ug/kg(Fig 1e). In vitro PTH activation of Tob expression in osteoblasts was also dose dependent and saturating at 10nM (Fig 1f). PTH activation of Tob mRNA expression was inhibited by a transcription inhibitor, DRB while it was still observed in the presence of protein synthesis inhibitor, cyclohexamide, revealing direct transcriptional events(Fig 1g). PTH treatment enhanced the levels of Tob protein expression in osteoblasts based on Western blot(Fig 1h).
To address functional meaning of PTH enhancement of Tob in bone, wild type and Tob knockout mice were subjected to iPTH. We used newborn mice whose bone mass has been shown to respond to PTH in a shorter period of time. Ten daily injections of PTH increased the crowdedness of cancellous bone as well as bulk of cortical bone in wild type as known before (Fig 2a, d). In contrast, Tob deficiency enhanced PTH-induced increase in cancellous bone volume (Fig 2a, b), cortical bone mass (Fig 2d, e) and trabecular thickness (Fig 2c). Histological examination showed Tob deficiency effects on PTH anabolic actions as well (Fig 2f). Histochemical analysis indicated that Tob deficiency suppressed the PTH-induced increase in the TRAP positive cell appearance (Fig 2g) and quantification indicated that Tob deficiency suppressed PTH-induced increase in osteoclast surface (bone surface covered by TRAP positive osteoclasts) (Fig 2h). Tob-deficiency did not largely appear to affect PTH-induced increase in osteoblast number (Fig 2i). To address molecular aspects of the effects of Tob deficiency, mRNA was isolated from bone and subjected to qRT-PCR. Interestingly, Tob deficiency did not largely alter the expression levels of osteoblast-related markers such as osteocalcin, Runx2, osterix, type I collagen and osteocyte-related marker including DMP1 or cross talk molecules for instance Sema3a. In contrast, Tob deficiency almost completely suppressed PTH-induced increase of the levels of Rankl mRNA and RANKL/Opg ratio in bone of mice subjected to 10 daily injections of PTH (Fig 2j, k).

As for the mechanism we examined the effects of Tob deficiency on PTH-dependent acute RANKL gene expression in bone in vivo at one hour. Tob deficiency almost completely suppressed PTH-induced acute RANKL expression in vivo (Fig 2l). Immunocytochemistry showed that PTH treatment also induced nuclear translocation of Tob-protein (Fig 2m). To examine the role of Tob in the regulation of RANKL promoter activity, siRNA was used to knockdown Tob and its effect on forskolin-induced RANKL promoter was examined. Tob knockdown suppressed forskolin-induced 2k proximal RANKL promoter activity revealing that Tob is required at least in part for adenylate cyclase activation that is downstream to PTH (Fig 2n). Tob knockdown by siRNA also suppressed PTH-induced luciferase activity driven by the 713 bp conserved non-coding region that is located 73kb upstream to the transcription start site (Fig. 3o).

Finally, we tested the relevance of the effects of Tob on PTH anabolic action in the repair of bone injury. We chose bone marrow injury model in that femoral cancellous bone is removed by inserting a 0.6mm diameter file through inter-condyle region. In this model, bone repair begins in 2-3 days reaching maximum bone regeneration by one week followed by bone resorption for another one week. To examine the resorption phase during the repair of bone injury, we examined the bone regeneration on day 11 of the injury (10 daily injections after injury, Fig 3a). PTH increased bone repair and regeneration slightly in wild type (Fig 3a arrows indicate ablated area; b shows 3D pictures of newly formed bone in ablated region). In contrast, Tob deficiency significantly enhanced PTH-induced repair and the mass of regenerated bone after bone injury (Fig 3c).

**Discussion:** We discovered that PTH enhances Tob gene expression in bone in vivo and osteoblasts in vitro. Furthermore, Tob is acting as a suppressor of anabolic action of PTH on bone mass in vivo. This suppression of bone mass was due to Tob requirement for PTH-induced RANKL expression via its promoter. Thus, PTH and Tob form a novel endogenous regulatory feedback loop. As a mechanism, Tob is required for PTH-induced RANKL expression in vivo and Tob is involved in forskolin-induced RANKL promoter activation. From pathological point of view, PTH-induced repair of bone injury is enhanced by Tob deficiency. This may lead to development of Tob inhibitors to enhance PTH actions in bone repair.
**Significance:** Novel regulatory mechanism underlying PTH anabolic action is discovered by identifying Tob as a PTH target. Tob is required for RANKL induction and thus reducing PTH effects on bone mass and repair. This may serve as a clue to develop new therapeutic measures for bone injury in combination with PTH.
ORS 2015 Annual Meeting

Paper No: 0027