Increased CKIP-1 within Osteoblast Suppress BMP Signaling to Inhibit Bone Formation During Aging

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Introduction: Aging-induced reduction in osteoblastic bone formation decreases bone mass, impairs bone architecture and subsequently increases bone fracture risk in aged patients. It has been demonstrated that dysregulated protein ubiquitylation plays an important role in aging-associated degenerative diseases [1]. A series of experimental evidences showed that several molecules involved in the ubiquitination process could modulate osteoblastic bone formation [2, 3]. However, there still lacks of ubiquitination-related molecules identified from human bone specimens, which could be responsible for the aging-induced reduction in osteoblastic bone formation. Recently, we have firstly found casein kinase-2 interacting protein-1 (CKIP-1, encoded by PLEKHO1), as an important ubiquitination-related molecule, specifically targets the linker region between the WW domains of smad ubiquitination regulatory factor 1 (Smurf 1, one member of the Hect family of E3 ubiquitin ligases), thereby augmenting its affinity for and promoting ubiquitylation of Smad 1/5 [4]. The Smad 1/5 are key molecules in signal transduction of bone morphogenetic protein (BMP) signaling and play a crucial role in regulating osteoblastic bone formation [5]. However, the exact pathological role of CKIP-1 within osteoblasts in aging-induced bone formation reduction is still not well elaborated.

Methods: Firstly, we collected bone specimens from 20 aged female patients with fracture (60~69 yr group (n=6), 70~79 yr group (n=8) and 80~89 yr group (n=6)). The intra-osseous mRNA expression of CKIP-1 and ALP were determined by real-time PCR, and the protein expression of phosphorylated Smad1/5 (p-Smad1/5) was detected by western blot. Thereafter, we performed ovariectomy (OVX) on 30 female S-D rats at 4 months of age, and sacrificed them 9, 13 and 17 months after OVX (n=10 for each time point), respectively. The 5th lumbar vertebral body (LV5) were subjected to real-time PCR analysis of the Ckip-1 mRNA expression, and western blot analysis of the CKIP-1, Smurf1 and p-Smad1/5 protein expression, respectively. The left femora were subjected to undecalcified bone histology analysis. The right femora were subjected to cryo-section for laser-captured micro-dissection (LCM) to isolate ALP+ cells, in which the Ckip-1 mRNA expression were analyzed by real-time PCR, and immunofluorescence (IF) analysis for protein expression of CKIP-1 and p-Smad1/5 in ALP+ cells,
respectively. The bilateral tibiae were pooled together at each time point for isolating ALP+ cells derived from compact bone by magnetic-activated cell sorting (MACs) for immunoprecipitation (IP) of total Smad1/5 and the subsequent western blot analysis for the ubiquitination of Smad 1/5. Moreover, we created mice carrying the mutant allele with LoxP sites bordering exon 3 to exon 6 of Ckip-1 gene (Ckip-1 flox) and crossed Ckip-1flo/x mice with Osx;Cre mice to generate osteoblast-specific Ckip-1 knockout mice (Osx;Ckip-1flo/x). Eighteen female Osx;Ckip-1flo/x mice and 18 female Ckip-1flo/x mice were ovariectomized at 4 months old, respectively. The mice in each group were sacrificed at 9 and 19 months of age (n=9 at each time point), respectively. The left femora were isolated for micro-CT followed by undecalcified bone histology analysis to determine the bone structure and bone formation level. The right femora were isolated for IF analysis to detect the expression of p-Smad1/5 in ALP+ cells. The bilateral tibiae were pooled together at each time points for MACs as described above to isolate ALP+ cells for IP of total Smad1/5 and western blot analysis for the ubiquitination of Smad 1/5.

Results: In human bone specimens, we found that the expression of intra-osseous CKIP-1 mRNA was increased with age, whereas the expression of ALP mRNA and the protein level of p-Smad1/5 were both decreased with age (Fig. 1a b & c). Moreover, the CKIP-1 mRNA level was negatively correlated with the ALP mRNA level and the p-Smad1/5 protein level during aging, respectively, while the p-Smad1/5 protein level was positively correlated with the ALP mRNA level during aging (Fig. 1d). Further, in OVX rats, we observed age-related decrease in either the width between xylenol and calcein labeling or osteoid, respectively (Fig. 2a). Consistantly, in the aged OVX rats, we also documented that the protein expression of CKIP-1 in whole-bone tissue was increased with age, respectively, whereas the protein level of p-Smad1/5 but not Smurf1 was decreased with age (Fig. 2b). In addition, we also observed increased number of cells co-expressing ALP and CKIP-1 at the distal femur in aged OVX rats during aging (Fig. 2c). Accordingly, the expression of Ckip-1 mRNA in osteoblasts (ALP+ cells) at distal femora isolated by LCM was also increased with age in aged OVX rats (Fig. 2c). We further detected increased ubiquitination level of total Smad5 in osteoblasts (ALP+ cells derived from the compact bone of tibiae and isolated by MACs) in the aged OVX rats during aging (Fig. 2d, similar result was found for Smad1 and data not shown). Moreover, we also observed decreased number of cells co-expressing ALP and p-Smad1/5 at distal femora of the aged OVX rats during aging (Fig. 2e). Next, we generated the osteoblast-specific Ckip-1 knockout mice (Osx;Ckip-1flo/x) (Fig. 3a). These mice showed absence of Ckip-1 mRNA in osteoblasts, and significantly lower expression of CKIP-1 protein in bone tissue when compared to those from control (Ckip-1flo/x) mice or non-skeletal tissues, respectively (Data not shown). The aged Osx;Ckip-1flo/x mice showed better organized micro-architecture of the trabecular bone at distal femur than the aged Ckip-1flo/x mice at each time point after OVX. In addition, the age-related deterioration in micro-architecture of the trabecular bone was obviously attenuated in aged OVX Osx;Ckip-1flo/x mice (Fig. 3b). Consistently, the age-related decrease in the width between xylenol and calcein labeling, which was observed in aged OVX Ckip-1flo/x mice, was remarkably attenuated in aged OVX Osx;Ckip-1flo/x mice (Fig. 3b). Furthermore, we found that the age-related increase in the ubiquitination level of total Smad5 in osteoblast (compact bone-derived ALP+ cells isolated by MACs), which was found in aged OVX Ckip-1flo/x mice, was obviously attenuated in aged OVX Osx;Ckip-1flo/x mice (Fig. 3c, similar result was found for Smad1 and data not shown). In addition, IF analysis showed age-related decrease in the number of cells co-expressing ALP and p-Smad1/5 at distal femora of aged Ckip-1flo/x mice after OVX, whereas the decrease was notably attenuated in aged Osx;Ckip-1flo/x mice after OVX (Fig. 3d).
**Discussion:** Aberrantly increased expression of CKIP-1 within osteoblasts could suppress BMP signaling to inhibit bone formation during aging.

**Significance:** This is the first study elaborating the contribution of the ubiquitination-related molecule, CKIP-1, to the pathophysiological mechanism of age-related bone formation reduction.