Regulation of osteoblast differentiation by interleukin-11 via AP-1 and Smad signaling

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Abstract. Mechanical stress and parathyroid hormone (PTH) are major stimulators, and aging and glucocorticoids excess are important suppressors of osteoblast differentiation. Mechanical stress and PTH stimulate interleukin (IL)-11 expression in cells of osteoblast lineage by enhancing transcription of IL-11 gene via an increase in intracellular Ca²⁺. The elevated Ca²⁺ activates extracellular signal-regulated kinase (ERK) to enhance phosphorylation of cyclic AMP response element-binding protein (CREB), which binds to the fosB gene promoter and enhances ∆FosB expression. ∆FosB dimerizes with JunD on the IL-11 gene promoter to enhance its transcription. Both mechanical stress and PTH also stimulate phosphorylation of Smad1 via an activation of protein kinase Cδ (PKCδ). Phosphorylated Smad1 binds to the IL-11 gene promoter and forms complex with ∆FosB/JunD to further enhance IL-11 gene transcription. The increased IL-11 then suppresses expression of Wnt inhibitors, including Dickkopf 1 (Dkk1) and 2, and enhances Wnt signaling to stimulate osteoblast differentiation and inhibit adipocyte differentiation. The suppression of osteoblast differentiation by aging involves a decrease in IL-11 gene transcription by a reduction in JunD binding to the activator protein (AP)-1 site of the IL-11 gene promoter. Glucocorticoids inhibit transcriptional activation of IL-11 gene by an interaction of glucocorticoid-glucocorticoid receptor (GR) complex with ∆FosB/JunD heterodimer. Thus, factors that enhance osteoblast differentiation stimulate, and those which suppress osteoblast differentiation inhibit IL-11 gene transcription, and IL-11 enhances Wnt signaling by suppressing expression of its inhibitors. These observations are consistent with the notion that IL-11 mediates stimulatory and inhibitory signals of osteoblast differentiation by affecting Wnt signaling.

Key words: Mechanical stress, Parathyroid hormone, Aging, Glucocorticoid, Wnt signaling

OSTEOBLASTS undergo differentiation from their progenitor cells of mesenchymal origin, and exert their osteogenic functions in a differentiation-dependent manner. Thus, osteoblasts synthesize type I collagen from the early differentiation stage, then secrete proteoglycans, osteonectin, alkaline phosphatase and other matrix proteins. Terminally differentiated osteoblasts synthesize osteocalcin and form matrix vesicles, which accumulate calcium and phosphate to form hydroxyapatite crystals. Osteocalcin controls mineralization of bone matrix by regulating the orderly deposition of hydroxyapatite to gap zone of type I collagen fibrils. These osteogenic processes must take place sequentially, and therefore osteoblast differentiation is tightly regulated by various systemic and local factors. Among systemic factors, parathyroid hormone (PTH) and sex steroids positively regulate, and glucocorticoids negatively regulate osteoblast differentiation. Bone morphogenetic proteins (BMPs) are important local factors to enhance osteoblast differentiation. Other than these soluble factors, mechanical stress is one of the most important regulators of osteoblast differentiation. In addition, aging causes refractoriness to most of the osteogenic stimuli and suppresses bone formation (Fig. 1). In this review, we summarize current understanding of the regulation of osteoblast differentiation by various stimuli and inhibitors, with special focus on the role of interleukin (IL)-11 in mechanical stress and PTH-induced stimulation, along with aging and glucocorticoid-induced suppression of osteoblast differentiation.
Senescence and bone formation

Osteoblasts originate from mesenchymal stem cells, and osteoblast progenitor cells can also give rise to adipocytes. Thus, lineage determination between osteoblasts and adipocytes is a critical component in the regulation of osteoblastogenesis [1]. Consistent with such a reciprocal regulation, it has been shown that osteoblastogenesis is suppressed while adipogenesis is enhanced in the aged bone marrow. Thus, deregulated differentiation of bipotential stromal cells may account for a decrease in the number of osteoblastic cells by aging.

Using senescence-accelerated mice (SAMP6), we demonstrated that these mice exhibit early decrease in bone mass and a reduction in bone turnover, that both osteoblastogenesis and osteoclastogenesis are suppressed and adipogenesis is enhanced from bone marrow stromal cells of SAMP6 mice, and that the expression of IL-11 is decreased in SAMP6 bone marrow stromal cells [2]. IL-11 is expressed in bone marrow stromal cells and is involved in the regulation of multiple biological processes such as enhancement of myeloid cell growth [3, 4], inhibition of adipogenesis [3, 5], and stimulation of osteoclast formation [6]. In an effort to clarify the mechanism of aging-associated bone loss, the effect of IL-11 on the differentiation of bone marrow cells from SAMP6 mice was examined. The impaired formation of both osteoblasts and osteoclasts was restored and the enhanced adipogenesis was suppressed by the addition of IL-11 [2]. Other cytokines that activate gp130 as a common signal transducer, IL-6 and leukemia inhibitory factor, did not have such effects. These results suggested that the reduction in IL-11 expression contributes to the impairment of osteoblastogenesis and osteoclastogenesis not only in senescence-accelerated model mice but also in the aging-related bone loss. In fact, transgenic mice overexpressing IL-11 exhibit increased bone mass with increased bone formation without any change in bone resorption, and are protected from aging-associated bone loss [7].

Senescence-associated change in \textit{IL-11} gene transcription

\textit{IL-11} gene promoter analysis revealed that two tandem activator protein (AP)-1 sites upstream of TATA box play a critical role in \textit{IL-11} gene transcription. Gel shift analysis showed that binding activity to the AP-1 site of the \textit{IL-11} gene promoter was reduced in nuclear extracts of stromal cells from SAMP6 mice. Among multiple components of AP-1 transcription factors, JunD binding to the AP-1 site was particularly decreased in SAMP6 mice. Furthermore, decreased JunD binding and IL-11 expression by stromal cells was similarly observed in aged mice of ICR strain [8]. Therefore, decreased AP-1 activity and a resultant decline in IL-11 expression by bone marrow stromal
cells appear to play a role in impaired bone formation by aging (Fig. 2). However, neither JunD mRNA nor JunD protein was reduced by aging [8]. From these results, it is plausible to assume that aging-related post-translational modification of JunD protein causes the reduction in the binding activity of JunD to the AP-1 site of the \textit{IL-11} gene promoter. Further studies are needed to clarify the mechanism of the reduction in JunD binding activity to the AP-1 site by aging.

**Glucocorticoids and bone formation**

Glucocorticoids excess causes a suppression of bone formation as well as an enhancement of bone resorption, and glucocorticoid-induced osteoporosis is one of the most common and serious complications of glucocorticoids excess. While the enhancement of bone resorption is more pronounced during the early phase, the inhibition of osteoblast differentiation along with enhancement of osteoblast apoptosis becomes more pronounced in the later phase of glucocorticoids excess [9]. Rauch et al. reported that mutant glucocorticoids receptor (GR) with disruption of GR dimerization interacts with AP-1 transcription factors to inhibit AP-1-induced stimulation of cytokine expression including \textit{IL-11} [10]. Our recent observations also demonstrate that glucocorticoids suppress osteoblast differentiation and enhance apoptosis via a suppression of \textit{IL-11} expression, and that glucocorticoid-GR complex interacts with \DeltaFosB/JunD heterodimer on the \textit{IL-11} gene promoter to suppress \textit{IL-11} gene transcription (Kuriwaka-Kido R, \textit{et al.} manuscript in preparation). These results demonstrate that the suppression of osteoblast differentiation is associated with a reduction in \textit{IL-11} gene transcription, that glucocorticoids suppress \textit{IL-11} gene transcription without dimer formation of glucocorticoid-GR complex, and that the reduction in \textit{IL-11} may play a role in the suppression of bone formation by glucocorticoids excess.

**Mechanical stress signaling and bone formation**

Mechanical stress to bone plays an important role in the maintenance of bone homeostasis. Mechanical unloading by prolonged bed rest, immobilization, or microgravity in space causes a marked loss of bone due to an imbalance between bone formation and resorption. While enhanced bone resorption in the endosteal surface is a major feature of unloading-induced bone loss in mature animals and humans [11], the impairment of bone formation in the periosteal surface constitutes an important mechanism for unloading-induced bone loss especially in the growing stage [12-14]. Therefore, it is important to understand the mechanism whereby mechanical loading enhances and unloading reduces bone formation.

In order to respond to mechanical stress, cells need to be equipped with mechanosensors. Fluid flow along cell surfaces produces fluid shear stress (FSS) and stress-generated electric potential, but cells appear to be more sensitive to FSS than to electric potential [15]. FSS causes an activation of stress-activated cation channel (SA-Cat), which causes an influx of extracellular Ca\(^{2+}\) to increase intracellular Ca\(^{2+}\). In addition, stretch of cell surfaces produces tensile stress, caus-
ing changes in integrins and cytoskeletal proteins [16] (Table 1). Among them, FSS is shown to be one of the most important signal transduction mechanisms to enhance osteoblast differentiation and bone formation in response to mechanical loading to bone [17, 18].

Activation of SA-Cat by FSS causes an increase in 
\( \text{Ca}^{2+} \) influx, which activates extracellular signal-regulated kinase (ERK), causing phosphorylation and activation of cyclic AMP response element-binding protein (CREB) [19-21]. Phosphorylated CREB binds to CRE region of the \( \text{fosB} \) gene promoter, and rapidly enhances \( \text{fosB} \) gene transcription [22]. Alternative splicing of \( \text{fosB} \) gene causes some splice variants, including FosB, \( \Delta \text{FosB} \) and \( \Delta \text{2FosB} \). Among them, \( \Delta \text{FosB} \) heterodimerizes with JunD on the \( \text{IL}-11 \) gene promoter, and enhances \( \text{IL}-11 \) gene transcription. Overexpression of some members of Fos family proteins including \( \Delta \text{FosB} \), Fra-1 and Fra-2 is shown to enhance bone formation [23, 24], although there is no direct evidence to indicate that the effect of overexpressed Fos family proteins is mediated via \( \text{IL}-11 \) expression in osteoblasts.

Increased intracellular \( \text{Ca}^{2+} \) also enhances adenosine triphosphate (ATP) release from cells, which activates G protein-coupled ATP receptors, ionotropic P2X7 and metabotropic P2Y receptors. P2X7 stimulates prostan glandin E2 (PGE2) synthesis. PGE2 signaling via EP4 receptor is shown to enhance bone formation [25]. Because EP4-mediated signals also activate CREB signaling [26], this signal may merge with \( \text{Ca}^{2+}-\text{ERK-CREB-Fos}^\Delta \) signal cascade. In contrast, P2Y activates phospholipase C (PLC) which induces inositol triphosphate (IP\(_3\)) release to stimulate \( \text{Ca}^{2+} \) efflux from intracellular stores [27, 28]. The increase in the intracellular \( \text{Ca}^{2+} \) can activate constitutive nitric oxide synthase (cNOS) to increase NO production (Table 1). NO is a strong inhibitor of bone resorption, and is reported to suppress the expression of receptor activator of nuclear factor-\( \kappa B \) ligand (RANKL) and increases osteoprotegerin (OPG) expression in bone marrow stromal cells [29]. Therefore, the increase in NO production may be important for the suppression of bone resorption by mechanical stress.

### PTH and bone formation

Another important stimulator of bone formation is PTH. Although continuous elevation of PTH predominantly enhances bone resorption, intermittent elevation of PTH stimulates osteoblast differentiation and bone formation with less stimulation of osteoclastic bone resorption. Thus, daily subcutaneous injections of PTH dramatically increase new bone formation along with old bone resorption, resulting in an increase in spine, hip and total body bone mass with an increase in bone strength in animals [30-32] and in osteoporotic patients [33, 34].

Precise mechanism of how the balance between bone formation and bone resorption changes by a difference in the length of PTH stimulation is currently unknown. However, there are interesting observations that, with the shortening of the time to peak PTH concentration (Tmax) and the half-life of circulating PTH, the overall effect becomes more anabolic under daily administration. Thus, daily nasal spray of PTH(1-34) gives a short Tmax, which increases bone formation markers and at the same time reduces bone resorption markers with net positive effect [35]. Similarly, weekly subcutaneous injections of PTH(1-34) causes an increase in bone formation markers with a reduction in bone resorption markers [36]. In contrast, although PTH-Fc fusion protein shows a long half-life, weekly or twice-weekly administration can give net anabolic effect on bone [37]. From these results, it is plausible to speculate that the relationship between the half-life of机械刺激对成骨细胞早期反应

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of PTH and the length of interval between doses determines the balance of the anabolic and catabolic actions of PTH on bone.

As to the mechanism of the enhancement of bone formation by PTH, O’Brien et al. demonstrated that mice expressing a constitutively active PTH receptor in osteocytes exhibit increased bone mass and bone remodeling, reduced expression of Wnt antagonist, sclerostin, with enhanced Wnt signaling. Interestingly, deletion of low-density lipoprotein receptor-related protein 5 (LRP5) attenuates the increase in bone mineral density (BMD), but does not affect the increase in bone remodeling by the transgene [38]. These observations suggest that the anabolic effect of PTH appears to be mediated via a suppression of the expression of canonical Wnt signal inhibitor, sclerostin, in osteocytes, which causes an activation of canonical Wnt signaling. In contrast, the effect of PTH on bone remodeling is independent of sclerostin-LRP5 pathway in osteocytes.

PTH also suppresses Dickkopf 1 (Dkk1) expression, another Wnt signal inhibitor, in osteoblasts. Guo et al. created mice overexpressing Dkk1 in osteoblasts, and examined whether the suppression of Dkk1 in osteoblasts is essential for PTH-induced activation of Wnt signaling and bone formation. The results demonstrate that PTH can still activate Wnt signaling despite overexpression of Dkk1 [39]. Thus, although PTH-induced activation of canonical Wnt signaling plays an important role for the anabolic effect of PTH, the relationship between osteocyte-mediated and osteoblast-mediated effects of PTH, as well as that between the inhibition of osteocyte-derived sclerostin and of other Wnt inhibitors from osteoblasts and osteocytes remain to be clarified.

### Regulation of IL-11 expression by mechanical stress and PTH

Mechanical unloading causes a marked reduction in the expression of IL-11 mRNA in the unloaded bone, and mechanical loading causes a rapid and robust increase in IL-11 mRNA expression in the loaded bone [21]. Recently, we found that not only mechanical stress but also PTH enhances the expression of IL-11 in osteoblasts both in vitro and in vivo (Kuriwaka-Kido R, et al. manuscript in preparation). These observations are consistent with the notion that the enhanced IL-11 expression may mediate the stimulation of bone formation in response to mechanical stress and PTH, whereas a reduction in IL-11 expression by aging may play a role in the age-related reduction in bone formation as mentioned previously.

### Mechanism of the regulation of IL-11 gene transcription

One of the earliest responding factors induced by mechanical loading in bone cells is an AP-1 family transcription factor, c-Fos [40]. However, because ubiquitous overexpression of c-Fos in transgenic mice develops osteosarcoma without evidence for increased bone formation [41], c-Fos is unlikely to be a factor mediating mechanical stress to bone formation. Mechanical loading to bone in vivo and FSS to osteoblasts in vitro also induces fosB gene transcription with a similar time course to that in c-fos gene transcription. The increase in fosB gene transcription is mediated via Ca²⁺-ERK-CREB signaling pathway, and activated CREB stimulates fosB gene transcription through binding to a putative CRE site in fosB gene promoter (Fig. 3) [22]. Enhanced fosB gene transcription by mechanical stress causes an accumulation of mainly ΔFosB protein, a short splice variant of FosB lacking C-terminal transactivation domain [42]. Because ΔFosB protein has long half-life which enables this protein to accumulate after transient stimulations [43], ΔFosB can be a suitable mediator of intermittent mechanical loading signal to sustained bone formation signal.

**IL-11** gene promoter contains two tandem AP-1 sites located upstream TATA box which confer transcriptional activation by transforming growth factor (TGF)-β and other stimuli [44]. Of the two AP-1 sites, site-directed mutagenesis revealed that 5' upstream AP-1 site confers the transcriptional activation of **IL-11** gene by mechanical stress. Mechanical loading enhances the expression of ΔFosB, and the upregulated ΔFosB forms heterodimers on the 5’AP-1 site of the **IL-11** gene promoter with JunD. JunD is bound to the 5’AP-1 site regardless of mechanical stimuli. Binding of the ΔFosB/JunD heterodimer to the 5′AP-1 site of **IL-11** gene promoter causes an enhanced transcription of **IL-11** gene [21]. Thus, down-regulation of ΔFosB/JunD expression by small interfering RNA (siRNA) reduces and overexpression of ΔFosB/JunD enhances **IL-11** gene promoter activity in osteoblasts [21].

BMPs play pivotal roles in the regulation of osteoblast differentiation and bone formation [45, 46]. However, the role of BMPs in mediating mechanical stress signal to osteogenic signal is controversial. Although
Matsumoto et al.

Mechanical stress induces fosB gene transcription by FSS was mediated via tyrosine phosphorylation and activation of protein kinase Cδ (PKCδ) in osteoblasts [51].

Mouse IL-11 gene promoter contains a putative Smad-binding element (SBE) along with the AP-1 sites. Studies with site-directed mutagenesis at the putative SBE and 5'AP-1 sites revealed that both SBE and AP-1 sites were required for full activation of IL-11 gene promoter activity by FSS. FSS-activated Smad1 is bound to SBE and forms a complex with ΔFosB/JunD heterodimer, which is bound to the 5’AP-1 site on the IL-11 gene promoter (Fig. 4) [51]. These observations demonstrate that Ca\(^{2+}\)-ERK-CREB-ΔFosB signaling and PKCδ-Smad1/5 signaling pathways merge together on the IL-11 gene promoter, and that AP-1 and Smad signaling cooperatively stimulate IL-11 gene expression in response to mechanical stress.

**Wnt signaling and IL-11**

Wnt/β-catenin signaling pathway plays an important role in the regulation of bone formation [53]. LRP5 and LRP6 are co-receptors for Wnt with the frizzled family of receptors, and are involved in signaling through the canonical Wnt/β-catenin pathway [54,.
Osteoblast differentiation and IL-11

Inactivating mutations in LRP5 result in osteoporosis pseudoglioma syndrome [56], and gain of function mutations in LRP5 gene gives rise to a high bone mass phenotype in humans [57-59] as well as in mice [60, 61]. As to the mechanism of the stimulation of bone formation by Wnt/β-catenin signaling, canonical Wnt signaling shifts cell fate of mesenchymal precursor cells toward the osteoblast lineage by induction of the osteoblastogenic transcription factors such as Runx2 and osterix, and suppression of the adipogenic transcription factors CCAAT enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) [62].

Wnt/β-catenin signaling is also a physiological response to mechanical loading, and mechanical unloading enhances and mechanical stress suppresses the expression of sclerostin, an inhibitor of Wnt/β-catenin signaling expressed in osteocytes [63]. These results suggest that modulation of sclerostin expression offers a finely tuned regulatory system in which osteocytes coordinate regional osteogenesis in areas with mechanical stress. However, the upstream signal that reduces sclerostin expression in response to mechanical stress remains unknown.

Wnt/β-catenin signaling can be inhibited by not only sclerostin but also Dkk1 and 2. Using murine primary osteoblasts that do not express sclerostin but express Dkk1 and 2, we demonstrated that FSS enhances Wnt/β-catenin signaling by suppressing Dkk1 and 2 expression [21]. The reduction of Dkk1 and 2 is mediated via the increased expression of IL-11 by mechanical stress, and knockdown of IL-11 expression by siRNA enhances and overexpression of IL-11 suppresses Dkk1 and 2. These observations demonstrate that stimulation of Wnt/β-catenin signaling is a major pathway mediating mechanical stress signal to osteogenic signal, and that enhanced IL-11 expression by mechanical stress is at least one of the upstream signals to enhance Wnt/β-catenin signaling (Fig. 5).
JunD heterodimer to further enhance IL-11 gene transcription. The increased IL-11 then suppresses the expression of Wnt inhibitors, including dickkopf (Dkk)-1 and 2, causing a stimulation of Wnt signaling and osteoblast differentiation.

**Conclusions**

There is a reciprocal regulation in the lineage determination of osteoblasts and adipocytes, and the lineage determination can regulate osteoblast differentiation. Mechanical stress and PTH are major stimulators, and aging and glucocorticoids excess are two important suppressors of osteoblast differentiation. Mechanical stress and PTH stimulate, while aging and glucocorticoids excess suppress IL-11 gene transcription in cells of osteoblast lineage. Signal transduction from mechanical stress and PTH stimulation involves increased intracellular Ca\(^{2+}\)-ERK-CREB pathway which enhances ΔFosB expression, and ΔFosB/JunD binds to the AP-1 site of the IL-11 gene promoter to enhance its transcription. Mechanical stress and PTH also stimulate Smad1 phosphorylation, which binds to the SBE site on the IL-11 gene promoter and forms complex with ΔFosB/JunD heterodimer to further enhance IL-11 gene transcription. The increased IL-11 then suppresses the expression of Wnt inhibitors, and enhances Wnt signaling. The suppression of osteoblast differentiation by aging and glucocorticoids is associated with a decrease in IL-11 gene transcription. Thus, stimulators of osteoblast differentiation enhance and inhibitors of osteoblast differentiation suppress IL-11 gene transcription. Taken together, these observations are consistent with the notion that IL-11 mediates stimulatory and inhibitory signals of osteoblast differentiation by affecting the Wnt signaling.

**Appendix**

We declare that we have no conflict of interest in connection with this paper.

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