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REVIEW

Rapamycin and the transcription factor C/EBPβ as a switch in osteoclast differentiation: implications for lytic bone diseases

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Abstract Lytic bone diseases and in particular osteoporosis are common age-related diseases characterized by enhanced bone fragility due to loss of bone density. Increasingly, osteoporosis poses a major global health-care problem due to the growth of the elderly population. Recently, it was found that the gene regulatory transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) is involved in bone metabolism. C/EBPβ occurs as different protein isoforms of variable amino terminal length, and regulation of the C/EBPβ isoform ratio balance was found to represent an important factor in osteoclast differentiation and bone homeostasis. Interestingly, adjustment of the C/EBPβ isoform ratio by the process of translational control is downstream of the mammalian target of rapamycin kinase (mTOR), a sensor of the nutritional status and a target of immunosuppressive and anticancer drugs. The findings imply that modulating the process of translational control of C/EBPβ isoform expression could represent a novel therapeutic approach in osteolytic bone diseases, including cancer and infection-induced bone loss.

Keywords C/EBPβ · MafB · Osteoporosis · Rapamycin · Cancer · Leukemia

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Introduction

Regulating bone mass, bone turnover (remodeling), and bone integrity requires a tight coupling between the two major bone cell types, the bone-forming osteoblasts, and the bone-resorbing osteoclasts [1]. Maintenance of a correct balance between the functions of both cell types is crucial during the phase of skeletal development and retaining bone homeostasis during adulthood. Pathological bone loss may occur when the cross talk between osteoblasts and osteoclasts is disturbed. This is often due to increased osteoclastic activity and diminished osteoblast activity, as observed in age-related and secondary osteoporosis or in inflammation-induced (e.g., in rheumatoid arthritis) or cancer-induced bone loss (reviewed in [2]). Although therapeutic strategies to treat osteoporosis have been developed, increasing knowledge on the regulation of osteoclastogenesis may facilitate identification of novel therapeutic targets and rational approaches.

Recently, a novel player in the regulation of osteoclast differentiation has been identified as the transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) [3]. C/EBPβ was found to affect both osteoblast activity in bone formation and osteoclast activity in bone resorption. These insights were obtained by combining studies of recombinant genetic mouse models and cell biological, pharmacological, and gene expression-profiling approaches. Briefly, the distinct C/EBPβ isoforms were found to differentially regulate expression of the downstream transcription factor MafB that acts like a brake to restrict osteoclastic differentiation and osteolytic activity [4]. C/EBPβ is normally expressed as two long transactivator isoforms, termed "LAP*/LAP" or as a truncated repressor, termed "LIP" (for the sake of simplicity, we will not discriminate between both activator isoforms, although LAP* displays additional

epigenetic features in comparison to LAP). The balance between LAP*/LAP and LIP is adjusted by the mammalian target of rapamycin kinase (mTOR) pathway, which acts as a sensor to integrate growth and nutrient stimuli. The LAP C/EBPβ isoform enhances expression of MafB that subsequently blunts osteoclastogenesis, whereas the truncated LIP C/EBPβ isoform enhances osteoclastogenesis by decreasing expression of MafB [3].

C/EBPβ

The transcription factor C/EBPβ belongs to the gene regulator family of the CCAAT/enhancer binding proteins, which consists of six members: α, β, δ, γ, ε, and ζ. C/EBPs are characterized by highly conserved carboxyl terminal "basic leucine zipper domains" (bZip), containing an alpha helical basic DNA binding domain and a leucine zipper coiled-coil dimerization domain. The N-terminal domains of C/EBPs are more variable and only partially conserved. The two main members C/EBPα and C/EBPβ are partially redundant during embryogenesis, and after birth, are involved in cell growth, proliferation, and differentiation in several tissues [5]. Both C/EBPα and C/EBPβ are encoded by intronless genes and are present as single messenger RNAs (mRNAs). Nevertheless, amino terminally truncated protein isoforms may be generated by a process known as alternative translation initiation [6]. Thus, the protein isoforms display different domains and exert distinct biological functions [7].

Alternative initiation of C/EBP translation is mediated by small "upstream open reading frames" (uORF) that act as regulatory elements to sense the activity of the translation machinery and to direct initiation to alternative start codons. A key signaling component of regulated translational initiation is the mTOR. The activity of mTOR depends on nutritional signals, including glucose and amino acid availability, and it also responds to extracellular signals such as growth factors and insulin [8]. The macrolide antibiotic rapamycin specifically inhibits the mTOR kinase, lowering the activity of the critical eukaryotic translation initiation factor 4E (eIF4E). Low eIF4E activity abolishes translation reinitiation, and in the case of C/EBPβ, diminishes expression of LIP. This results in a higher LAP to LIP ratio of the C/EBPβ isoforms and alters expression of C/EBPβ target genes (Fig. 1) [6].

C/EBPβ is involved in the differentiation of a large variety of cell types, including keratinocytes, hepatocytes, mammary epithelial cells, ovarian luteal cells, adipocytes, B cells, and macrophages. Moreover, C/EBPβ regulates cell survival, apoptosis, metabolism, inflammation, and tumorigenic transformation [5, 7, 9]. C/EBPβ is expressed as three different protein isoforms differing in their N-terminal

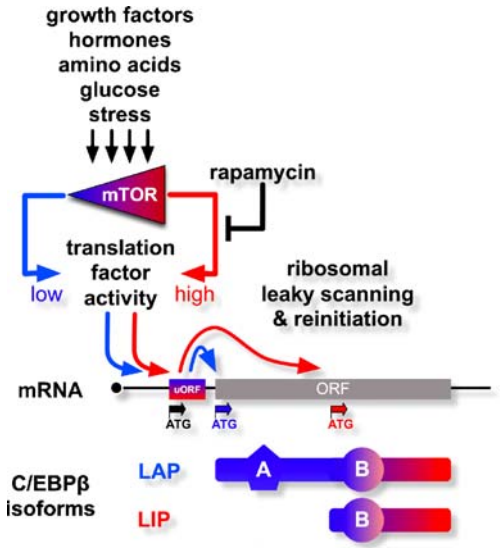

Fig. 1 Translational control and C/EBPβ isoform regulation. Growth factor, hormone, nutrition, and stress signals are integrated by the mammalian mTOR kinase that monitors the growth and differentiation status of a cell. The mTOR kinase (low activity (*blue*); high activity (*red*)) regulates the function of the translation initiation machinery. The cis-regulatory upstream open reading frame uORF in the C/EBPβ mRNA senses the activity of the translation machinery and directs initiation, leaky scanning, and reinitiation. High activity of the translation machinery directs reinitiation and production of the truncated "repressor" isoform LIP of C/EBPβ. Lowering the activity of the initiation machinery results in leaky scanning over the uORF ATG and translation initiation at the first ATG, resulting in the production of the long "activator" isoform LAP. Rapamycin inhibits mTOR signaling, resulting in the production of mainly the LAP isoform

domains, LAP*, LAP (both are transcriptional activators), and LIP (a transdominant repressor), each displaying unique functions in various cellular processes. The diversity of C/EBPβ's functional properties is further increased by signaling-dependent posttranslational modifications that regulate the biological activity and the subcellular distribution of C/EBPβ [7]. Action of C/EBPβ requires dimerization through the bZip domain, either with another C/EBPβ molecule or through heterodimerization with any of the other C/EBP family members or some transcription factors with compatible bZip domains.

C/EBPβ controls bone cells

C/EBPβ has been suggested to act as a scaffold protein in the assembly of osteogenic transcription factors [9], such as with Runx2 or ATF4, thereby enhancing osteoblast differentiation [10, 11]. Analyses of C/EBPβ-deficient mice confirmed a role of C/EBPβ in osteoblast differentiation and bone formation: In the absence of C/EBPβ, bone mass is decreased [12] due to decreased osteoblast differentiation

and function [11]; however, this appeared to entail also noncell autonomous effects [3, 13]. C/EBPβ deficiency also resulted in growth retardation during embryogenesis [11, 14] and at postnatal stages [3], due to diminished chondrocyte differentiation [11, 14]. C/EBPβ activates the cyclin-dependent kinase inhibitor $p57^{kip}$ in chondrocytes that restricts chondrocyte proliferation and stimulates the transition to hypertrophic differentiation [14].

Surprisingly, expression of the (repressor) LIP isoform instead of the entire C/EBPβ gene from its endogenous locus in "knock-in" mouse mutants restored bone formation and rescued the growth retardation observed in C/EBPβ-deficient animals [3]. Moreover, LIP enhanced osteoblast differentiation and function [3], possibly by acting as a coactivator for Runx2 [10].

Besides cell autonomous effects, C/EBPβ is possibly also involved in paracrine action and the coupling between osteoblasts and osteoclasts. C/EBPβ and its related family member C/EBPδ induce IGF-I expression [15–18] that represents an important anabolic factor for bone formation. Similar to TGFβ [19], IGF-I is released from the bone matrix by osteoclasts and may then stimulate osteoblasts [20]. In addition, coupling between osteoclasts and osteoblasts is thought to involve the cytokines interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) [20] that also represent targets of C/EBPβ in osteoclasts [3].

C/EBPβ as a switch in osteoclast differentiation

The first observations suggesting a connection between C/EBPβ and osteoporosis were made over a decade ago in connection with the cytokine IL6. C/EBPβ, in synergy with NFκB, was found to act downstream of estrogen, involving binding of the estrogen receptor and resulting in suppression of IL6 gene transcription in osteoblasts [21]. This is of clinical relevance as decreased estrogen levels are one of the major causes of postmenopausal osteoporosis, which results in increased IL-6 that activates osteoclasts and thus bone resorption [22, 23]. Indeed, C/EBPβ-deficient mice show a similar general pathology as mice overexpressing IL6 [24] and with bone loss as a consequence of enhanced activity of osteoclasts [3]. Macrophage defects observed in C/EBPβ-deficient mice [25] were the first indication of a possible role of C/EBPβ in the bone-resorbing osteoclast, which shares a monocytic precursor cell with macrophages.

In contrast to macrophages, osteoclast precursors fuse into large polykaryons to generate mature osteoclasts that attach to the bone surface and resorb bone matrix. Both differentiation and osteolytic activity of osteoclasts requires macrophage colony-stimulating factor (M-CSF) and membrane-bound receptor activator of NFκB ligand (RANK-L), which are both produced by osteoblasts and by stromal cells. Osteoblasts also produce osteoprotegerin, a decoy receptor of RANK-L, thereby counteracting and fine tuning the effect of RANK-L in osteoclastogenesis. These cytokines activate downstream osteoclastic transcription factors, including NFATc1, Mitf, and c-Fos that affect proliferation, differentiation, and survival of osteoclasts [2, 26].

Absence of C/EBPβ or expression of only the LIP isoform was observed to strongly enhance osteoclast differentiation. This suggested that the absence of LAP would cause enhanced osteoclastogenesis, and LAP would induce a gene whose product blocks osteoclast differentiation. Such an inhibitor was identified as MafB, a transcription factor recently found to be involved in osteoclastogenesis [4] and a target gene of LAP [3].

MafB is also a bZIP transcription factor that, however, belongs to a distinct family. MafB is important in several developmental processes [27] and in oncogenesis [28]. In the hematopoietic system, the function of MafB is restricted to the monocytic lineage [29]. Similarly to C/EBPβ, the expression of MafB starts at the myeloblast stage and strongly increases during macrophage differentiation [29]. Although not strictly required for macrophage differentiation, absence of MafB in monocytes results in an altered phenotype. Absence of MafB reduces F4/80 levels in macrophages [30]. Upon M-CSF treatment, MafB-deficient macrophages form multiple extensive cellular extrusions, often branched, known as filopodia [31], a process that also occurs in osteoclasts and that is required for cell migration [32].

MafB directs macrophage versus osteoclast differentiation [4], and recently, MafB was reported to restrict M-CSF receptor-induced activation of the PU.1 gene [33] that encodes an early and essential myeloid and osteoclastic transcription factor [26]. No bone phenotype has been reported of MafB knockout mice so far, possibly due to their early postnatal lethality [27]. However, the MafB protein interacts with and thereby attenuates the activity of the osteoclastogenic transcription factors NFATc1, Mitf, and c-Fos [4]. The long C/EBPβ isoform LAP induces MafB expression and thus inhibits osteoclastogenesis, whereas absence of LAP (such as in C/EBPβ-deficient animals or in knockin mutants expressing only the inhibitory LIP isoform) results in diminished MafB expression. Lack of MafB enhances the functionality of NFATc1, its downstream target OSCAR, and probably other factors, including Mitf and c-Fos, to augment expression of osteoclast genes, including the cell fusion-promoting genes DC-STAMP, ATP6v0d2, and TNFα [34]. The opposing roles of the different C/EBPβ protein isoforms in MafB expression suggest that the C/EBPβ isoform ratio plays a fundamental role as an upstream regulator in the initiation of osteoclastogenesis and connects MafB expression and osteoclastogenesis to the mTOR pathway (Fig. 2).

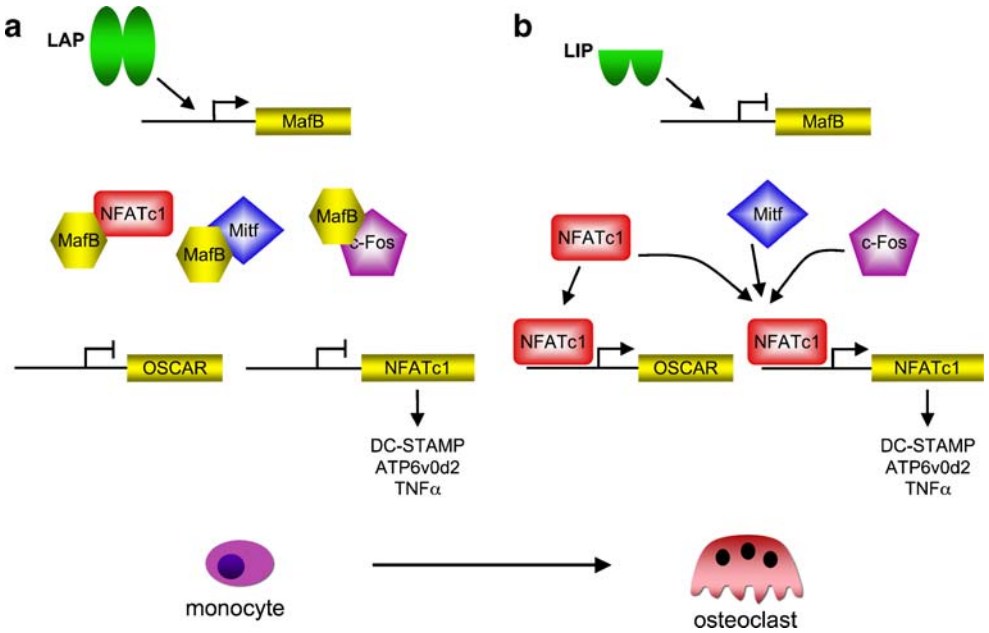

Fig. 2 C/EBPβ as a master switch in osteoclast differentiation. **a** The LAP isoform of C/EBPβ induces expression of MafB. MafB binds to and inactivates the osteoclastic transcription factors c-Fos, Mitf, and NFATc1. Inactivation of these key transcription factors prevents osteoclast differentiation by inhibition of osteoclast target gene expression of OSCAR and NFATc1, resulting in inhibition of NFATc1 target genes, including the cell fusion genes DC-STAMP, ATP6v0d2, and TNFα (partially derived from [4, 34]). **b** The LIP isoform of C/EBPβ inhibits MafB expression. As a result, MafB becomes limiting, and lack of MafB permits access of the osteoclast transcription factors (often in conjunction with NFATc1 as a major osteoclast transcription factor) to activate target genes and osteoclast differentiation

Implications for osteolytic diseases

Therapies of osteoporosis concentrate on the inhibition of the pathological bone resorption by osteoclasts using mainly bisphosphonates, but also estrogen-like drugs such as raloxifene, or strontium ranelate [1, 2, 35]. However, bisphosphonates inhibit the bone remodeling cycle, raising the need for the development of shorter-acting resorption inhibitors, which will not affect the bone-remodeling process itself too much to ensure continuous repair of

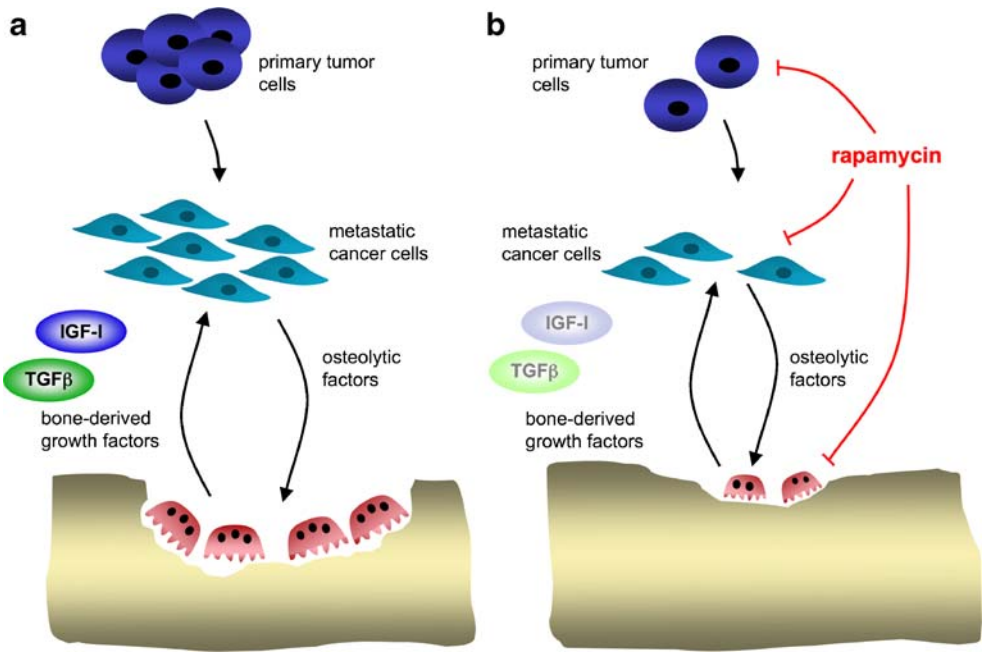

Fig. 3 Dual effect of rapamycin on cancer-induced bone loss. **a** In this hypothetical model, several types of tumor cells with high LIP expression (breast, prostate, lung, multiple myeloma) preferentially generate osteolytic metastasis by activating osteoclasts to release tumor-promoting growth factors (e.g., *TGFβ*, *IGF-I*). This results in a continuous cycle of stimulation of metastatic cells and bone resorption (derived from [56]). **b** The vicious circle between tumor and stroma (osteoclasts) may be interrupted by drugs that impinge on translational control, such as rapamycin or its derivatives

microfractures and prevent additional weaknesses of the skeleton [2]. Therefore, novel therapeutic strategies are required to improve treatment strategies in osteoporosis and other lytic bone diseases. The findings of involvement of translational control of C/EBPβ isoforms in directing osteoclastogenesis may entail such novel targets for therapy. Translational control is affected by the antibiotic rapamycin that specifically inhibits mTOR signaling, resulting in a shift of the C/EBPβ isoform ratio toward the LAP isoform (Fig. 1). Rapamycin treatment thus favors LAP expression over LIP, which results in enhanced MafB gene activation and thus inhibition of both osteoclastogenesis and bone resorption. Inhibition of mTOR by rapamycin inhibits osteoclastogenesis not only in mouse [3, 36] but also in human cells [37], suggesting that the murine model may provide disease relevant data. Importantly, a derivative of rapamycin has already been shown to inhibit bone loss in an experimental rat model, where osteoporosis was induced by ovariectomy [37]. So far, however, no clinical data is available concerning the impact of rapamycin on bone. Taken together, these data suggest that rapamycin could potentially serve as a therapeutic agent in treating osteolytic diseases [38].

The mTOR signaling pathway is also important in the regulation of autophagy, a process recently proposed to be involved in osteoclast function [39, 40]. Rapamycin induces autophagy and therefore might play a role in late osteoclastogenesis. This would be in addition to the action of rapamycin early in osteoclast differentiation, where it inhibits differentiation [3]. Autophagy was suggested to decelerate aging processes [41], as also recently found for rapamycin that extends the life span of aged mice [42]. This may suggest that dampening mTOR signaling prevents age-related disease progression, including cancer.

Along these lines, the LIP isoform has been associated with enhanced proliferation of multiple myeloma and breast cancer and in Hodgkin and anaplastic large cell lymphoma. A rapamycin derivative was demonstrated to decrease tumor cell proliferation by abrogating LIP expression [43]. These observations further strengthen the notion that C/EBPβ is an important downstream target of mTOR, affecting cell proliferation and differentiation in diverse cell types. Moreover, rapamycin has been shown to inhibit tumor cell metastasis in an osteosarcoma mouse model [44]. Rapamycin has already been considered as a novel treatment in multiple myeloma, where it restrains tumor cell proliferation [45] and has antitumor activity, as reported in breast cancer patients where rapamycin was tested in clinical phase trials [46]. Induction of autophagy by rapamycin would, in addition, sensitize cancer cells to complementary treatments [47]. Both multiple myeloma and breast cancer generate bone metastases, causing local osteolytic lesions [48, 49]. Also, in rheumatoid arthritis, local bone loss occurs, and rapamycin was found to be beneficial due to its immunosuppressant characteristics [50, 51]. The observation that rapamycin also functions as an antiresorptive agent by modulating the C/EBPβ isoform ratio [3] may suggest a bipartite function [52] as tumor suppressor or immune suppressant and as an osteoclast regulator, as depicted in Fig. 3.

Rapamycin's immunosuppressant action due to modulation of regulatory T cells might raise concerns when used for other purposes than immunosuppression, e.g., as in cancer patients or elderly with osteolytic diseases. Problems in wound healing [53] and occurrence of anemia [54] have been reported, although no increase in infectious complications are also reported [55].

It is interesting to note that the downstream C/EBPβ target MafB has also been found to be deregulated in the majority of multiple myeloma cases [28]. Moreover, C/EBPβ is known to play a central role in inflammation. Both processes have a strong impact on osteoclastogenesis and can induce pathological bone resorption. The recent identification of the role of the C/EBPβ isoform ratio in the control of osteoclast differentiation and bone resorption may link the pathology of these lytic bone diseases to the translational control of a distinct gene regulator and may thus open new avenues for novel therapeutic approaches.

The usage of rapamycin to direct translational control might have several potential benefits to treat osteolytic-associated diseases, as it can attack these diseases at different levels. Rapamycin treatment, combined with restraining osteoclast differentiation, may have combinatorial functions in treating osteolytic diseases. However, one has to take into consideration that inhibition of the mTOR pathway could have adverse side effects, requiring the development of novel rapamycin analogs or novel tissue-specific mTOR inhibitors displaying less side effects.

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