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REVIEW ARTICLE

Molecular mechanisms of BMP-induced bone formation: Cross-talk between BMP and NF-κB signaling pathways in osteoblastogenesis

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Summary Osteoblasts are bone-forming cells that differentiate from mesenchymal stem cells. Differentiation processes are coordinately and dynamically controlled in the mesenchymal cells by specific signal transduction pathways. Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, induce not only bone formation *in vivo*, but also osteoblast differentiation of mesenchymal cells *in vitro*. BMP signals are transduced from plasma membrane receptors to the nucleus through both Smad-dependent and -independent pathways, and are regulated by many extracellular and intercellular proteins that interact with BMPs or components of BMP signaling pathways. To understand the molecular mechanisms underlying the role of BMPs in osteoblast differentiation, it is important to elucidate the BMP signaling transduction pathways that are active during osteoblast differentiation. In this review, we summarize the BMP signaling pathways that are known to function in osteoblast development. We also describe our recent findings regarding the molecular mechanisms underlying the cross-talk between BMP/Smad and NF-κB pathways in osteoblast differentiation.
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1. Introduction

Despite its hard structure, bone actually exists in a constant state of dynamic turnover known as bone remodeling in order to maintain bone volume and calcium homeostasis throughout life [1,2]. At remodeling sites, osteoblasts lay down new bone, while osteoclasts resorb existing bone. Osteoblasts produce bone matrix proteins such as type I collagen, the most abundant (90%) extracellular matrix protein in bone. The remaining 10% is composed of a large number of non-collagenous proteins, e.g., osteonectin, osteocalcin, bone sialoprotein and various proteoglycans. Non-collagenous proteins participate in the process of matrix maturation or mineralization, and may regulate the functional activity of bone cells [1,2]. Osteoblasts, chondrocytes, myocytes and adipocytes all derive from a common progenitor known as the mesenchymal stem cell [3,4]. During the process of differentiation, progenitor cells acquire specific phenotypes under the control of respective regulatory factors [1—4].

Bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF-β) superfamily, were originally identified by their ability to induce ectopic bone formation when implanted into muscle tissue [5,6]. BMPs are involved in nearly all processes associated with skeletal morphogenesis and play pivotal roles in the regulatory signaling network. BMP signals are transduced from plasma membrane receptors to the nucleus through both Smad-dependent and -independent pathways, and are regulated by many extracellular and intracellular molecules that interact with BMPs or components of BMP signaling pathways. The unique and specific ability of BMPs to induce bone formation was expected to be useful for the development of bone regeneration treatments; however, BMPs cannot generate enough of a clinical response to be used in this context [7—9]. A possible reason might be that inflammatory cytokines inhibit the bone formation and osteoblast differentiation induced by BMPs. For example, one inflammatory cytokine, tumor necrosis factor (TNF) α, inhibits osteoblast differentiation in multiple models, including fetal calvaria, bone marrow stromal cells and MC3T3-E1 cells [10—12].

Inflammatory cytokines such as TNFα and interleukin (IL)-1 are produced primarily by activated macrophages, but are also produced by many other structural cell types, including fibroblasts, smooth muscle cells and osteoblasts [13,14]. The binding of these cytokines to their receptors results in the activation of an inflammatory response that is classically mediated by a broad variety of pro-inflammatory cytokines, including interleukins, interferon-γ and chemokines [13,14]. In addition, intracellular signal transduction pathways activated by these cytokines elicit a wide spectrum of other cellular responses. These responses include the modulation of differentiation and proliferation in multiple cell types, and the induction of apoptosis via several signaling pathways, such as meiosis-specific serine/threonine protein kinase (MEK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase and NF-κB [14].

Among these intracellular molecules, the signal transduction stimulated by TNFα and IL-1 occurs partly through the activity of the NF-κB family of transcription factors [15,16]. In this pathway, the activated NF-κB, mainly p50 and p65 heterodimers, enters the nucleus and binds to NF-κB-responsive elements to regulate the expression of genes that are involved in the regulation of immune and inflammatory responses, proliferation, tumorigenesis and survival [15,16].

In contrast to NF-κB, BMP signaling provides anti-proliferative differentiation signals to osteoblasts as well as other tissues [17]. Furthermore, BMP, acting through both Smad-dependent and -independent mechanisms, inhibits the cell cycle and increases apoptosis by regulating pro-apoptotic proteins [18,19]. Together, there seem to be antagonistic effects between BMP/Smad and NF-κB signaling systems. Therefore, in this review, we will first summarize the role of BMP signaling in osteoblast differentiation and then discuss the molecular mechanisms underlying TNFα/NF-κB-mediated regulation of BMP-2-induced osteoblast differentiation.

2. Regulation of osteoblast differentiation

Bone consists of hydroxyapatite crystals and various extracellular matrix proteins, including type I collagen, osteocalcin, osteopontin, bone sialoprotein and proteoglycans. Most of these bone matrix proteins are secreted and deposited by mature osteoblasts, which are aligned on the bone surface. The formation of hydroxyapatite crystals in osteoid is also regulated by osteoblasts [20—22]. Expression of a number of bone-related extracellular matrix proteins, high alkaline phosphatase (ALP) enzymatic activity and responsiveness to osteotropic hormones and cytokines are believed to be major characteristics of osteoblasts.

During embryogenesis, bone tissues are formed through two distinct pathways: intramembranous ossification and endochondral ossification. In the case of intramembranous ossification, osteoblasts differentiate directly from mesenchymal cell considensations, whereas in the case of endochondral ossification, the condensed mesenchymal cells differentiate into chondrocytes and form a cartilaginous template. Then the surrounding mesenchymal cells immediately differentiate into osteoblasts after maturation of hypertropic chondrocytes in the template suggesting that osteoblasts and chondrocytes are derived from a common

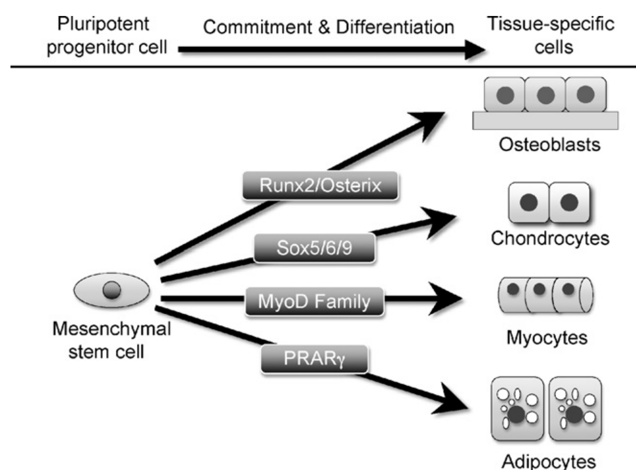

Figure 1 A schematic model for differentiation of a mesenchymal stem cell into tissue specific cells by specific transcriptional factors. A mesenchymal stem cells can differentiate into osteoblasts, chondrocytes, myoblasts, and adipocytes. Each differentiation program is regulated by specific transcription factors: Runx2/Osterix, Sox5/6/9, MyoD family and PPARγ, respectively.

progenitor cell (Fig. 1). Furthermore, it is well known that osteoblasts, chondrocytes, adipocytes, myoblasts, tendon cells, and fibroblasts differentiate from the common precursor of bone marrow mesenchymal stem cells [4,5,23]. The lineages are determined by different transcription factors. The transcription factors Runx2, Osterix and β-catenin regulate osteoblast differentiation, Sox family proteins (Sox9, Sox5 and Sox6) regulate chondrocyte differentiation, MyoD transcription factors (MyoD, Myf5 and Myogenin) regulate myogenic differentiation, and C/EBP family (C/EBPβ, C/EBPδ and C/EBPα) and PPARγ transcription factors regulate adipocyte differentiation (Fig. 1). Runx2 first directs multipotent mesenchymal cells to a preosteoblastic state, after which β-catenin, Osterix and Runx2 together convert the cells into mature osteoblasts [4,5,23].

Several cytokines and hormones, such as BMP, TGFβ, Wnt, hedgehog, fibroblast growth factors and estrogen, are involved in the regulation of mesenchymal cell differentiation by stimulating intracellular signaling pathways. Among them, BMP is one of the most powerful inducers of ectopic bone formation and strongly promotes differentiation of mesenchymal cells into osteoblasts [4,5,23].

3. The BMP/Smad signaling pathway

3.1. BMP ligands

BMPs are members of the TGF-β superfamily that were originally identified by their ability to induce ectopic bone formation when implanted into muscle tissue [5]. Subsequently, several cDNAs encoding proteins capable of ectopic bone formation were isolated and more than 15 BMP genes have now been identified in vertebrates [6]. Several recombinant BMP proteins have been shown to induce ectopic bone formation [5,24]. BMPs are translated as large preproproteins containing signal peptides. Proproteins undergo dimerization, after which specific proteolytic enzymes cleave the dimerized proprotein to generate biologically active dimeric mature proteins. It has been shown that some BMP heterodimers are more potent in inducing ectopic bone formation than their respective homodimers [5,24].

Although most BMPs are expressed in a variety of tissues during embryogenesis, some are detected in only a restricted set of tissues after birth. In adult mice, BMP-3, BMP-4, BMP-5 and BMP-6 are highly expressed in lung, whereas BMP-7 is highly expressed in kidney. The expression levels of BMP-4 and BMP-6 are much higher in adults than in young mice. Osteoblasts are an important source of BMPs in bone matrix, and expression of some BMP mRNAs is induced during bone formation. BMP-4 is transiently induced in callus-forming cells in the early phase of fracture healing. BMP-6 is highly expressed in hypertrophic chondrocytes, which are intermediate between cartilage and bone during endochondral ossification [24,25].

3.2. BMP receptors

Signaling by TGF-β superfamily members, including BMPs, is generally initiated upon their binding to transmembrane receptors. BMPs bind to two major types of membrane-bound serine/threonine kinase receptors, type I and type II receptors. BMPR-IA (ALK-3), BMPR-IB (ALK-6) and ActR-IA (ALK2) are BMP type I receptors. Constitutively active ALK1 has been shown to phosphorylate BMP-regulated Smads, suggesting that ALK1 is also BMP type I receptor. Individual BMP ligands bind to different type I receptors in different cell types. In general, type II BMP receptors show lower ligand binding ability and lower ligand specificity than those of type I receptors. ActR-II and ActR-IIB binds to BMP-2 and BMP-4 at least in *in vitro* [24,25]. The type II receptor, BMPR-II, binds exclusively to BMP ligands, including BMP-2, BMP-4, BMP-6, BMP-7, GDF-5 and GDF-9. ActR-II and ActR-IIB, originally identified as activin receptors, can also act as receptors for BMP-6, BMP-7 and GDF-5. BMP receptor oligomerization appears to be different from activin and TGF-β receptor oligomerization. For activin and TGF-β receptors, the ligands first bind to type II receptors, which in turn leads to the recruitment of type I receptors. However, it has been demonstrated that BMPR-II and type I receptors form both homomeric and heteromeric complexes, even before ligand stimulation [24,25].

BMPR-IA is more widely expressed than BMPR-IB among tissues, but BMPR-IB is the only receptor expressed in all

types of cartilage. Expression of the dominant-negative form of BMPR-IB, but not dominant-negative BMPR-IA, blocks chondrogenesis and osteogenic differentiation. ALK2 is expressed in isolated chick osteoblasts and chondrocytes, and overexpression of constitutively active ALK2 enhances chondrocyte maturation, suggesting that it may be essential for normal chondrocyte maturation and skeletal development [24,25].

3.3. BMP intracellular signal transduction

Smad proteins play central roles in intracellular signaling by members of the TGF-β superfamily. Upon binding of a BMP ligand, the type II receptor transphosphorylates the type I receptor at an intracellular juxtamembrane site termed the GS domain. The phosphorylated type I receptor, in turn, phosphorylates a set of intracellular substrate signaling proteins collectively known as Smads. Smad proteins are classified into three subgroups, i.e., receptor-regulated Smads (R-Smads), a common-partner Smad (Co-Smad) and inhibitory Smads (I-Smads). The BMP-specific R-Smads, Smad1, Smad5, and Smad8 are phosphorylated by the BMP type I receptors at their carboxy-terminal Ser-Ser-X-Ser motifs. The phosphorylated Smad proteins form complexes with the Co-Smad, Smad4, translocate into the nucleus, bind to the regulatory elements of target genes, and regulate their transcription (Fig. 2) [5,24,25].

Smads are critically involved in osteoblast differentiation. Smad1 and Smad5 have been shown to be the major signaling molecules for inducing differentiation of myoblastic C2C12 cells into osteoblasts. Smad1 and Smad5, but not Smad8, synergize with Smad4 to promote chondrocyte differentiation from chondroprogenitor cells. By contrast, Smad8 and Smad4 exhibit modest effects in mesenchymal cells.

Smad1 and Smad5, but not Smad8, are activated by BMP-6 and BMP-7 [26], whereas Smad1, Smad5 and Smad8 are all activated by BMP-2 [27]. Non-Smad signaling mediated by several kinases, including mitogen-activated protein (MAP) kinases p38, ERK and Jun N-terminal kinase (JNK), and phosphoinositol-3 (PI3) kinase, is also activated by BMP receptors and regulates cell differentiation [24,25].

3.4. Role of BMPs in osteoblast differentiation *in vitro* and *in vivo*

In order to examine the molecular mechanisms underlying ectopic bone-induction, the biological effects of recombinant BMP proteins on osteoblast differentiation have been studied *in vitro* using cell lines and primary cells. In cultures of osteoblast lineage cells, various BMPs induce the expression of ALP, parathyroid hormone (PTH)/PTH-related protein receptor, type I collagen and osteocalcin [28]. BMPs also stimulated the formation of mineralized bone-like nodules in these cells. BMPs can also induce osteoblast differentiation in several other types of cell in culture as well as some pluripotent cell lines [28]. C3H10T1/2 clone 8, a cell line established from a C3H mouse embryo, differentiates into myoblasts, adipocytes and chondrocytes in the presence of 5-azacytidine [29,30]. BMP-2 and BMP-4 induce osteoblastic differentiation of C3H10T1/2 cells [30–33].

BMPs were originally identified as an activity that induces ectopic bone formation in muscular tissue, suggesting that BMPs regulate the pathway of differentiation of myogenic cells. While examining this possibility, Katagiri et al. found that BMP-2 inhibited myogenic differentiation of a mouse myoblast cell line, C2C12, and instead diverted their differentiation pathway into that of osteoblasts [34]. TGF-β also inhibited myogenic differentiation of C2C12 cells, but failed to induce osteoblast differentiation [34]. This system has been widely used to examine the role of BMP signaling in osteoblast differentiation [3,24–26].

Smad transcription factors are substrates of activated type I receptor kinases in the cytoplasm. The phosphorylated Smad proteins translocate into the nucleus, bind to the

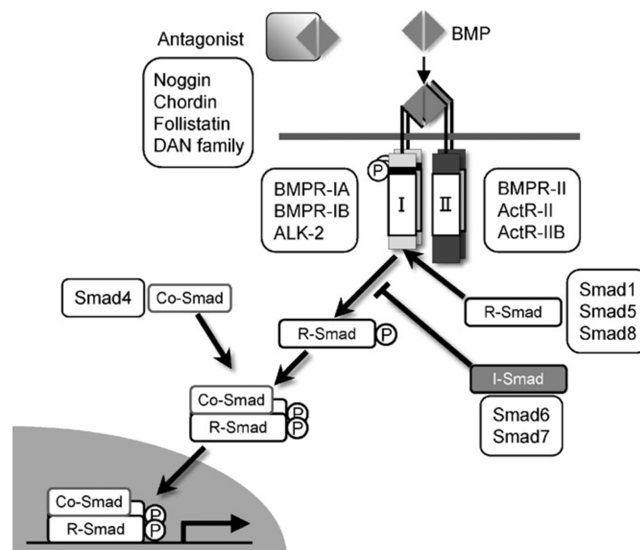

Figure 2 A schematic model for BMP signal transduction. Signaling of BMPs is initiated upon their binding to type I and type II transmembrane receptors, which are serine/threonine kinases. The type I receptors phosphorylate Smad1/5/8 from a complex with Smad4. The complex translocates into nucleus and associates with other DNA-binding proteins, and then regulates the transcription of the target genes.

regulatory regions of target genes and regulate their transcription [4,5,24,25]. Thus, Smad proteins are key molecules in the transduction of signals from the cell membrane to the nucleus. Overexpression of Smad1, Smad5 or Smad8 induces ALP activity and osteocalcin production in C2C12 and 10T1/2 cells [35]. Smad4 is a Co-Smad that cooperates with all R-Smads. By contrast, both Smad6 and Smad7 inhibit signal transduction of the all TGF-β superfamily members. Smad signals are regulated positively and negatively not only by other Smads but also by transcriptional activators and/or repressors [3,5,24,25].

Although BMP is known to be one of the most powerful cytokines to induce ectopic bone formation and strongly promotes differentiation of mesenchymal cells into osteoblasts and chondrocytes [24—26], until recently, it was still unknown whether BMP was really important for bone formation in physiological and pathological conditions, because mice lacking BMP-2 or BMP-4 die at early embryonic stages. However, mice often offer a unique opportunity to combine genetic and biochemical studies of skeletal growth and patterning. So far, at least 150 mouse loci are known that affect various aspects of skeletal morphogenesis. The mouse ''*short ear*'' gene is required for normal growth and patterning of skeletal structures, and for repair of bone fractures in adults. Kingsley et al. [36] showed that the *short ear* region contains the gene for BMP-5 that is deleted or rearranged in several independent mutations at the *short ear* locus. Furthermore, mutations in *Gdf-5* gene, another member of the TGF-β superfamily, are known to be responsible for the skeletal abnormalities observed in the classical mouse mutation, brachypodism, which are characterized by skeletal abnormalities restricted to the limbs and limb joints [37]. The skeletal elements of BMP-6 deficient mice are indistinguishable from wild-type. BMP-7-deficient mice also have skeletal patterning defects restricted to the rib cage, the skull, and the hindlimbs [38,39]. The BMP-4/7 double heterozygotes develop minor defects in two restricted areas of the skeleton, the rib cage, and the distal part of the limbs, suggesting that Bmp-4 and Bmp-7 act in the same pathway to assure proper guidance of mesenchymal condensations of the ribs extending toward the sternum [40]. Inactivation of the *Gdf-6* gene causes defects in joint, ligament, and cartilage formation at sites distinct from those seen in *Gdf-5* mutants, including the wrist and ankle, the middle ear, and the coronal suture between bones in the skull [41]. Mice lacking both *Gdf-5* and *Gdf-6* show additional defects, including severe reduction or loss of some skeletal elements in the limb, additional fusions between skeletal structures, scoliosis, and altered cartilage in the intervertebral joints of the spinal column [41]. Homozygous mutant mice carrying a targeted deletion of *Gdf-11* exhibit anteriorly directed homeotic transformations throughout the axial skeleton and posterior displacement of the hindlimbs [42]. The transgenic mice overexpressing the dominant-negative form of BMP type I receptor, which is necessary for BMP signaling, were generated [43]. The mice exhibited a severe reduction in bone formation, indicating an important role for BMP signaling in bone development [43]. By contrast, Bmp-3-deficient mice have twice as much trabecular bone as wild-type, indicating that BMP-3, the most abundant BMP in adult bone, is a negative determinant of bone density [44].

The mutations of BMP signaling have been identified in humans with heritable skeletal abnormalities. A mutation in human cartilage-derived morphogenetic protein (CDMP-1) is associated with a recessive human chondrodysplasia (acromesomelic chondrodysplasia, Hunter-Thompson type) [45]. The disorder, characterized by skeletal abnormalities restricted to the limbs and limb joints, is phenotypically similar to murine brachypodism, which is due to mutations in *Gdf-5*, the mouse homologue of hCDMP-1. By contrast, mutations in the human noggin gene and those in GDF/CDMP1 leading to its hyperactivation results in symphalangism [46]. Recently, mutations in the ALK2 gene have been identified in individuals with fibrodysplasia ossificans progressiva (FOP) [47—49]. These findings indicate that BMPs are key molecules of differentiation of osteoblasts and chondrocytes from mesenchymal progenitor cells during skeletal development.

BMP induces Runx2 expression in mesenchymal progenitors in a Smad-dependent fashion. Runx2 interacts with Smad1 and Smad5, and regulates the expression of target genes which are involved in osteoblast differentiation [50—54]. However, several molecules inhibit this signaling cascade, thereby suppressing osteoblast differentiation. For example, Smad6 negatively controls osteoblast differentiation as an inhibitory Smad [53]. Yoshida et al. showed that Tob, a member of the emerging family of anti-proliferative proteins, inhibits Smad1, Smad5 and Smad8, and consequently suppresses osteoblast differentiation. Furthermore, Tob-deficient mice showed greater bone mass resulting from increased numbers of osteoblasts [54]. Therefore, these inhibitory network systems also contribute to the differentiation of mesenchymal cells into osteoblasts.

3.5. Role of Runx2 and Osterix in osteoblast differentiation

Since Runx2-deficient mice have no bone tissue or osteoblasts, this transcription factor appears to be indispensable for osteoblast differentiation. Runx2 has also been identified as a gene responsible for cleidocranial dysplasia (CCD), an autosomal-dominant disease with abnormalities in the bone formed by intramembranous ossification. BMP-2 markedly upregulates the expression of Runx2 through the activation of Smad signaling [53,55,56]. Furthermore, Smad1 and Smad5 physically interact with Runx2 and enhance its transcriptional and osteoblastogenic activity [50,53]. The importance of this interaction was supported by a genetic report showing that mutations in Runx2 that delete the Smad-binding domain result in CCD [51]. Further evidence has established the significance of the BMP—Smad—Runx2 in osteoblast differentiation. As expected, Runx2-deficient mice show severely impaired osteoblast differentiation, but no abnormalities in the patterning of the skeleton. Although the overexpression of Runx2 promotes osteoblastic differentiation of mesenchymal cells [57], osteopenia and frequent bone fractures are observed in transgenic mice overexpressing Runx2 specifically in bone tissue [58]. Therefore, Runx2 appears to inhibit bone formation at the late stage.

In addition to Smad and Runx2, C/EBP family proteins, which play a key role in the adipocyte differentiation of mesenchymal cells, also regulate osteoblast differentiation. C/EBPβ and C/EBPδ associate with Runx2 and increase osteo-

calcin promoter activity [59], and C/EBPβ promotes osteoblast differentiation in association with Runx2 [60], providing a novel role for the C/EBP family in bone metabolism.

Another transcription factor, Osterix, was originally identified as a gene that was markedly upregulated by BMP-2 stimulation [61]. Like the Runx2 knockout mice, Osterix-deficient mice showed a complete absence of bone formation and osteoblasts, indicating that Osterix is also essential for bone development and osteoblastogenesis [61]. In these mice, Runx2 is still expressed in the mesenchymal cells, but Osterix is not expressed in the mesenchymal cells of Runx2-deficient mice [61]. These data suggest that Runx2 is an upstream transcription factor for Osterix. Furthermore, Osterix binds to NFATc1 (NFTA2) or NFATc2 (NAFT1) and increases the promoter activity of Col1α1 [62]. However, the target genes of Osterix are currently unknown. Identification of these target genes will provide new areas for exploring the molecular mechanisms of osteoblast biology.

4. Inflammatory cytokines suppress osteoblast differentiation

Inflammatory cytokines contribute to local and systemic bone loss in inflammatory bone diseases such as rheumatoid arthritis and periodontitis as well as in estrogen deficiency [63—65]. In patients with rheumatoid arthritis, TNFα and other cytokines are overproduced in inflamed joints by various cells that infiltrate the synovial membrane [65]. Anti-TNF drugs such as infliximab, etanercept and adalimumab have been shown to not only diminish signs and symptoms of disease, but also to prevent joint damage [66]. Under these conditions, osteoblast-mediated bone formation cannot compensate for accelerated osteoclastic bone resorption, suggesting a direct inhibitory effect of TNFα on osteoblasts. Further support for this inhibitory effect was provided by TNFα transgenic mice, which exhibit well-characterized features of rheumatoid arthritis and develop symptoms of general osteoporosis, including a reduction of trabecular bone in the metaphysis [67]. In addition, osteoblastic cells derived from TNFα transgenic mice form significantly fewer and smaller nodules under basal conditions and in the presence of BMP-2, again indicating that TNFα reduces osteoblast function.

Consistent with clinical and *in vivo* animal studies, the inhibitory effects of TNFα on bone formation *in vitro* were first reported in 1987 using a neonatal rat calvarial organ culture system [10]. Subsequent studies demonstrated that TNFα blocks osteoblast differentiation in multiple models, including fetal calvaria, bone marrow stromal cells and MC3T3-E1 cells [11,12]. TNFα inhibited not only spontaneous osteoblast differentiation, but also BMP-induced osteoblast differentiation via Smad-dependent or -independent mechanisms [52]. TNFα inhibited the BMP-2-induced expression of Runx2, osteocalcin and ALP activity in C2C12 cells in a dose-dependent manner. TNFα also inhibited the phosphorylation of Smad1/5/8 by inducing Smad6, an inhibitory Smad [68]. These responses were mediated via several signaling pathways, including MEK, ERK, JNK, p38 and NF-κB [14]. Our studies have focused on NF-κB signals, because BMP and NF-κB have opposite biological functions during inflammatory processes [15,16,18,19].

5. The NF-κB signaling pathway

The NF-κB/Rel family of ubiquitously expressed transcription factors consists of five members in mammals: p65 (RelA), c-Rel, RelB, NF-κB1 (p50/p105) and NF-κB2(p52/p100) [15,16]. All five members share an N-terminal domain of 300 amino acids, designated the Rel homology domain (RHD), which is responsible for DNA binding, dimerization and interaction with the inhibitory IκB proteins. Three members, p65, c-Rel and RelB, contain C-terminal transcriptional activation domains (TAD) that are crucial for their ability to induce target gene expression, while homodimers of p50 and p52 lack TADs and therefore have no intrinsic ability to drive transcription. p65, c-Rel and RelB are synthesized as mature proteins, whereas p50 and p52 are first synthesized as longer precursors, p105 and p100, respectively, which are then processed to their smaller forms by the proteasome (Fig. 3).

NF-κB activity is controlled by shuttling from the cytoplasm to the nucleus in response to cell stimulation. In

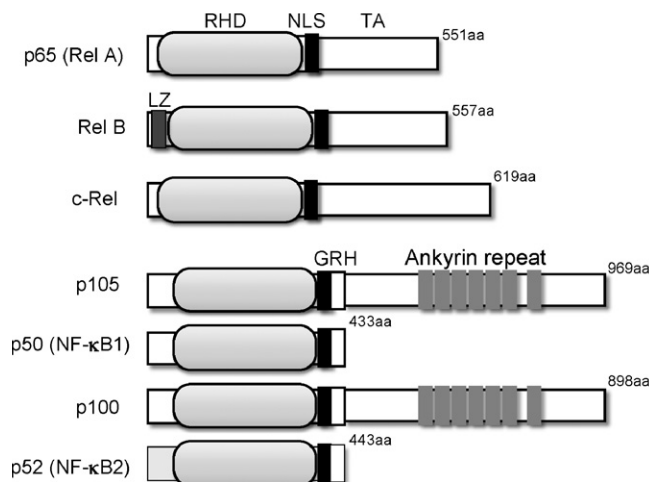

Figure 3 Schematic representation of NF-κB proteins family of proteins. Members of the NF-κB proteins families are shown. The number of amino acids in each protein is indicated on the right. Presumed sites of cleavage for p100 (amino acid 447) and p105 (amino acid 433) are shown. RHD: Rel homology domain, NLS: nuclear localization signal, TA: transcriptional activation domain, LZ: leucine zipper, GRH: glycine rich hinge.

unstimulated cells, NF-κB is predominantly localized in the cytoplasm in a complex with several inhibitory IκB proteins, including IκBα, IκBβ, IκBε and IκBγ. The IκB proteins are characterized by the presence of five to seven ankyrin repeats that are essential for their interaction with NF-κB proteins. In response to a variety of stimuli, such as TNFα or IL-1β, IκBs are phosphorylated (Ser32 and Ser36 for IκBα and Ser19 and Ser21 for IκBβ) by the activated IκB kinase (IKK) complex, leading to their ubiquitination and degradation by the 26S proteasome. This process, which is a key step in the regulation of the NF-κB pathway, allows NF-κB dimers to translocate to the nucleus, where they induce the expression of target genes (Fig. 4) [15,16].

The IKK complex consists of two catalytic kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, NEMO (NF-κB essential modulator), also called IKKγ [15,16]. IKKα and IKKβ share 52% amino acid homology and have similar functional domains, including an N-terminal catalytic domain, a centrally positioned leucine-zipper motif that is involved in homo- and heterodimer formation, and a C-terminal helix-loop-helix (HLH) domain. NEMO/IKKγ interacts with a C-terminal region of IKKβ, designated the NEMO binding domain (NBD), which is critical for the formation of the IKK complex [69]. Although NEMO/IKKγ has no catalytic function, it is indispensable for signal-dependent NF-κB activation. IKKβ is mainly required for the canonical NF-κB pathway that depends on IκB degradation. Despite the structural similarity of IKKα and IKKβ, biological and genetic studies indicate that IKKβ is the dominant kinase involved in IκBs phosphorylation. On the other hand, IKKα provides a partially redundant role in stimulus-induced NF-κB activation. Recent studies have shown that IKKα is involved in an alternative (non-canonical) NF-κB pathway that regulates the RelB/p52 dimer [15,16]. In unstimulated cells, RelB is retained in the cytoplasm as a RelB-p100 precursor complex, which prevents RelB-mediated transcriptional activity. Upon stimulation, the IκB-like C-terminal domain of p100 is proteolyzed, releasing RelB-p52 dimers that translocate to the nucleus. This non-canonical NF-κB pathway is induced in response to only a few members of the TNF family, including B cell-activating factor (BAFF), CD40 ligand and lymphotoxin β (LTβ), and is mostly involved in lymphoid organ development (Fig. 4) [15,16].

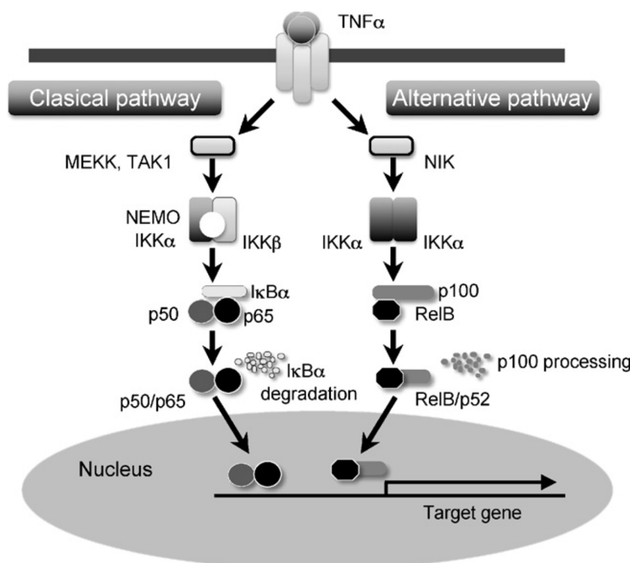

Figure 4 Two different NF-κB signaling pathways. The classical (canonical) pathway (left) is activated by a large number of agonists, such as TNFα, IL-1, LPS, or TCR. Activation of this pathway depends on the IKK complex (IKKα/β and NEMO), which phosphorylates IκBα (Ser32, 36) to induce rapid degradation. This pathway is essential for immune responses, inflammation, tumorgenesis and cell survival. The alternative (non-canonical) pathway (right) is activated by a limited number of agonists which are involved in secondary lymphoid organogenesis, mature B cell function and adaptive immunity. This pathway requires NIK and IKKα, which induce the slow processing of p52 from p100, resulting in dimerization and activation of the p52/Rel B heterodimer.

6. The role of NF-κB signaling in BMP-2-induced osteoblast differentiation

The transcription factor NF-κB has a key role in inflammation and immune responses [15,16]. NF-κB signaling may be associated with metabolic diseases such as cachexia and diabetes [70,71]. Inhibition of IKKβ-specific peptides suppresses inflammatory bone loss by inhibiting osteoclastogenesis in an arthritis model, suggesting that NF-κB is a major target of inflammatory bone diseases [72,73]. Although the involvement of NF-κB in osteoclastogenesis has been well investigated, little is currently known about the possible involvement of NF-κB in osteoblast differentiation, especially the cross-talk between BMP/Smad and NF-κB signaling in osteoblast differentiation.

Eliseev et al. reported that inhibition of NF-κB by overexpression of a dominant-negative form of IκBα (IκBαDN) leads to the induction of osteoblast differentiation [74]. Osteosarcoma Saos2 cells overexpressing IκBαDN (Saos2-IκBαDN) exhibit higher expression of osteoblast phenotypic genes such as ALP, Runx2 and osteocalcin, and are more responsive to BMP-2 relative to wild-type cells (Saos2-wt) or empty vector-infected controls (Saos2-EV). Furthermore, BMP-2 signaling and Smad phosphorylation are significantly increased in Saos2-IκBαDN cells in comparison to Saos2-EV cells. Inhibition of NF-κB signaling in Saos2-IκBαDN cells is associated with decreased expression of the BMP signaling inhibitor Smad7, suggesting that in osteosarcoma Saos2 cells, NF-κB represses BMP/Smad signaling and BMP-2-induced differentiation through Smad7 [74].

The role of TNFα-induced NF-κB activation in BMP-2-induced osteoblast differentiation has also been examined. Blocking the activation of NF-κB by TNFα using the cell permeable NF-κB activation antagonist TAT-NBD prevented TNFα from suppressing TGFβ-stimulated Smad luciferase activity, BMP-2-induced Runx2 mRNA expression and osteoblast differentiation in MC3T3-E1 cells. By contrast, an inactive mutant (MUT-TAT-NBD) had no effect. These results indicated that inhibition of NF-κB could suppress the anti-osteoblastogenic activity of TNFα and relieve the suppression of BMP-2-induced Runx2 expression mediated by TNFα [75].

We also showed that NF-κB is involved in the inhibitory effect of TNFα on BMP-2-induced osteoblast differentiation. Inhibition of BMP-2-induced Id1-luciferase activity coincides with the activation of NF-κB in p65−/− mouse embryonic fibroblasts (MEFs) in a p65 dose-dependent

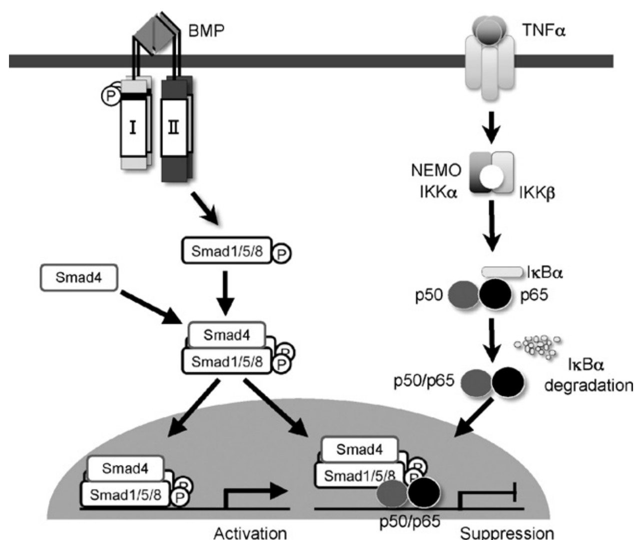

Figure 5 A model of NF-κB-mediated inhibition of BMP/Smad-mediated DNA-binding activity. NF-κB, particularly the p65 subunit, binds the Smad1/Smad4 complex directly or indirectly, and that this binding interferes with the DNA binding of Smad proteins induced by BMP-2.

manner. Overexpression of p65 completely abrogated the Id1-luciferase activity induced by the constitutively active form of Smad1 and Smad4. TNFα inhibited BMP-induced DNA binding of Smad proteins in wild-type MEFs, whereas TNFα failed to inhibit DNA binding in p65−/− MEFs. The pharmacological NF-κB inhibitor mentioned above restored the inhibitory effects of TNFα on both BMP-induced DNA binding of Smad proteins and ALP activity. This study suggests that NF-κB, particularly the p65 subunit, binds the Smad1/Smad4 complex directly or indirectly, and that this binding interferes with the DNA binding of Smad proteins induced by BMP-2 (Fig. 5, [76]).

Consistent with these results, time- and stage-specific inhibition of endogenous IKKβ in differentiated osteoblasts substantially increases trabecular bone mass and bone mineral density without affecting osteoclast activities in mice [77]. Inhibition of the IKKβ-NF-κB axis maintains bone formation, thereby preventing osteoporotic bone loss induced by ovariectomy in adult mice, and enhances the expression of Fos-related antigen (Fra-1), an essential transcription factor involved in bone matrix. These results strongly suggest that targeting IKKβ-NF-κB may help to promote bone formation in the treatment of osteoporosis and other inflammatory bone diseases [77].

7. Conclusion

Since the discovery of BMP in 1965, it has been expected to serve as a powerful agent of osteoanagenesis. Notably, BMP can potently induce ectopic bone when matrix is implanted into muscular tissue in rodents [5]; however, BMPs did not produce the desired results in either basic research with primates or at the clinical level [7–9].

The effects of BMPs are modulated by extracellular BMP antagonists, including noggin, chordin, follistatin, which block BMP signaling by binding to BMPs and preventing their association with specific cell surface receptors. Downstream effectors of BMP signaling, the Smad proteins, are also regulated by protein degradation via the ubiquitin-proteasome system. Smurf1 and Smurf2 are Smad-specific E3 ubiquitin ligases that selectively interact with BMP receptor-activated Smads [24,25]. Future identification of proteins that enhance rather than inhibit BMP/Smad signaling during osteoblast differentiation will prove a significant advance to the field.

Recently, we and another group revealed that the inhibitory effect of TNFα on BMP signaling in osteoblast differentiation is closely linked to the NF-κB pathway [72,73]. NF-κB activation inhibits osteoblast differentiation at least in part by interfering with Smad signaling. Smads 1/5/8 are generally accepted as very early downstream effectors in the BMP signaling pathway, and are thus fundamental for osteoblastic differentiation in response to BMP ligands [24,25]. An intracellular balance of signal intensities between NF-κB and BMP/Smad is therefore crucial for osteoblast differentiation, and might also inform future bone regeneration applications. Furthermore, modulation of Smad activity may be useful for establishing new approaches for the treatment of a variety of bone and cartilage disorders.

Conflict of interest statement

We declare no conflict of interest.

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