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REVIEW ARTICLE
Bone Biology

Regulatory mechanisms of osteoblast and osteoclast differentiation

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Bone is continuously destroyed and reformed to maintain constant bone volume and calcium homeostasis in vertebrates throughout their lives. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Recent developments in bone cell biology have greatly changed our conceptions of the regulatory mechanisms of the differentiation of osteoblasts and osteoclasts. Bone morphogenetic proteins (BMPs) play critical roles in osteoblast differentiation. The discovery of Smad-mediated signals revealed the precise functions of BMPs in osteoblast differentiation. Transcription factors, Runx2 and Osterix, are found to be essential molecules for inducing osteoblast differentiation, as indicated by the fact that both Runx2-null mice and Osterix-null mice have neither bone tissue nor osteoblasts. Smad transcriptional factors are shown to interact with other transcription regulators, including Runx2. Also, the recent discovery of receptor activator of NF-κB ligand (RANKL)–RANK interaction confirms the well-known hypothesis that osteoblasts play an essential role in osteoclast differentiation. Osteoblasts express RANKL as a membrane-associated factor. Osteoclast precursors that express RANK, a receptor for RANKL, recognize RANKL through the cell–cell interaction and differentiate into osteoclasts. Recent studies have shown that lipopolysaccharide and inflammatory cytokines such as tumor necrosis factor receptor-α and interleukin I directly regulate osteoclast differentiation and function through a mechanism independent of the RANKL–RANK interaction. Transforming growth factor-β super family members and interferon-γ are also shown to be important regulators in osteoclastogenesis. These findings have opened new areas for exploring the molecular mechanisms of osteoblast and osteoclast differentiation.
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Introduction

Bone is continuously destroyed and reformed in vertebrates in order to maintain bone volume and calcium homeostasis throughout their lives. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Osteoblasts produce bone matrix proteins including type I collagen, the most abundant extracellular protein of bone, and also take charge of mineralization of the tissue (Aubin and Triffitt, 2002). Osteoblasts, chondrocytes, myocytes and adipocytes are all derived from a common progenitor called undifferentiated mesenchymal cells. During the process of their differentiation, progenitor cells acquire specific phenotypes under the control of respective regulatory factors. Bone morphogenetic proteins (BMPs) play critical roles in the differentiation of undifferentiated mesenchymal cells into osteoblasts. Recent studies have elucidated the molecular mechanism of osteoblast differentiation induced by BMP.

Osteoclasts are multinucleated cells responsible for bone resorption. The most characteristic feature of osteoclasts is the presence of ruffled borders and clear zone (Väänänen and Zhao, 2002). Vacuolar H^+-ATPase exists in the ruffled border membrane of osteoclasts, and acidifies resorbing area under the ruffled border. The ruffled border is surrounded by a clear zone, which serves for the attachment of osteoclasts to the bone surface to maintain a microenvironment favorable for bone resorption. Osteoclasts are differentiated from hemopoietic cells of the monocyte/macrophage lineage

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under the control of bone microenvironments. Osteoblasts or bone marrow stromal cells have been shown to regulate osteoclast differentiation providing the microenvironment similar to bone. The recent discovery of new members of the TNF receptor-ligand family has clarified the molecular mechanism of osteoclast differentiation regulated by osteoblasts/stromal cells. This review article describes the current knowledge of the mechanisms of the regulation of osteoblast and osteoclast differentiation, which will deepen our understanding of oral biology and oral diseases.

Regulation of osteoblast differentiation

Characteristics of osteoblasts and their progenitors

Osteoblasts are specialized cells that function in bone formation in vertebrates. Bone tissue mainly consists of hydroxyapatite crystals and various kinds of extracellular matrix proteins including type I collagen, osteocalcin, osteonectin, osteopontin, bone sialoprotein and proteoglycans (Young *et al*, 1992; Robey *et al*, 1993; Mundlos and Olsen, 1997). Most of these bone matrix proteins are secreted and deposited by polarized mature osteoblasts, which are aligned on the bone surface. The formation of hydroxyapatite crystals in osteoid is also regulated by osteoblasts. Therefore, the expression of a number of bone-related extracellular matrix proteins, high enzyme activity of alkaline phosphatase (ALP), and responses to osteotropic hormones and cytokines are believed to be major characteristics of osteoblasts.

During embryogenesis, bone tissue is formed through two independent pathways: intramembranous ossification and endochondral ossification (Karsenty, 1999; Yamaguchi, komori and Suda, 2000). In both pathways, osteoblasts play unique roles in the bone formation. In the case of intramembranous ossification, osteoblasts are differentiated directly from mesenchymal cells in the mesenchymal condensation. On the other hand, in the endochondral ossification, mesenchymal cells differentiate into chondrocytes first and form a cartilaginous template. Then osteoblasts are differentiated from the surrounding mesenchymal cells immediately after maturation of hypertrophic chondrocytes in the template (Chung *et al*, 1998). These developmental processes of bone and cartilage suggest that osteoblasts and chondrocytes are derived from a common progenitor cell (Figure 1). Indeed, cell cultures prepared from calvaria or bone marrow show mixed populations of osteoblasts, chondrocytes, adipocytes and skeletal muscle cells. Some clonal embryonic fibroblast cell lines differentiate into multiple phenotypes of cells in response to treatment with 5-azacytidine (Taylor and Jones, 1979). The establishment of the pluripotent cell lines from the calvaria indicated that a pluripotent progenitor cell can differentiate into tissue-specific cells such as osteoblasts, chondrocytes, adipocytes and myoblasts (Grigoriadis, Heersche and Aubin, 1988, 1990; Yamaguchi and Kahn, 1991). The progenitor cells may acquire a tissue-specific phenotype concomitantly with losing their pluripotency under the control of various stimulants. Tissue-specific transcription factors regulate the differentiation of tissue-specific cells from the progenitor cells (Figure 1).

Discovery of BMPs

In 1965, Urist (1965) found that demineralized bone matrix contains a unique activity that induces ectopic bone when the matrix is implanted into muscular tissue. This activity was named 'BMP'. Subsequently, cDNAs encoding several active proteins for ectopic bone formation were isolated, and the proteins were eventually renamed 'BMPs' (Wozney *et al*, 1988). More than 15 genes of BMPs have been identified in vertebrates, and several recombinant BMP proteins have been shown to induce ectopic bone formation (Kingsley, 1994, 2001; Hogan, 1996; Wozney and Rosen, 1998; Reddi, 2001). Bone-inducing activity is unique to BMPs among the growth factors. It is believed that osteoblasts are cells responsible for the secretion and deposition of BMPs into the extracellular matrix during bone formation. BMPs, except BMP-1, belong to the transforming growth factor-β (TGF-β) superfamily, members of which are known to regulate the proliferation, differentiation and death of cells in various tissues (Hogan,

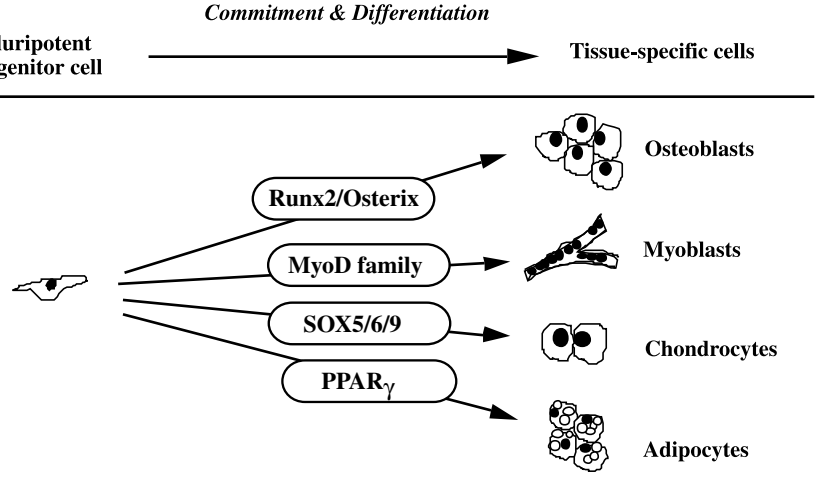

Figure 1 A schematic model for differentiation of a mesenchymal progenitor cell into tissue-specific cells. A pluripotent progenitor cell originated from undifferentiated mesenchyme can differentiate into several kinds of tissue-specific cells such as osteoblasts, myoblasts, adipocytes and chondrocytes. Each differentiation pathway seems to be regulated by tissue-specific transcription factors: Runx2/Osterix, MyoD family, PPARγ and SOX5/SOX6/SOX9, respectively

1996; Massague, 2000; Miyazono, ten Dijke and Heldin, 2000; Wrana, 2000). BMPs are also involved in the organogenesis of both hard and soft tissues (Kingsley, 1994, 2001; Hogan, 1996). Although TGF-β superfamily members have significant homology with each other, neither TGF-β nor activin/inhibin induces ectopic bone formation (Sampath, Muthukumaran and Reddi, 1987). BMPs are the only growth factors known at present to induce the whole process of ectopic bone formation in vertebrates.

Role of BMPs in skeletal development in vivo

The unique activity of BMPs suggests that they regulate osteoblast and chondrocyte differentiation during skeletal development. Identification of skeletal abnormalities in animals and patients with mutations in the BMP genes has confirmed this hypothesis. The first such example was the case of BMP-5 in mice (Kingsley *et al*, 1992). The mutant mouse 'short ear' has a defect in a gene required for normal growth and patterning of skeletal structures, and for repair of bone fractures in adults. Kingsley *et al* (1992) showed that the short ear region encodes BMP-5, which is deleted or rearranged in several independent mutations at the short ear locus. Storm *et al* (1994) reported that mutations in Gdf5, another member of the TGF-β superfamily, are responsible for skeletal alterations in brachypodism (bp) mice, which are characterized by skeletal abnormalities restricted to the limbs and limb joints. The human homolog of Gdf5, CDMP-1, has also been identified as a gene associated with a recessive chondrodysplasia, Hunter–Thompson type, which has a phenotype similar to that of bp mice (Thomas *et al*, 1996). Another mutation of CDMP-1 causes a chondrodysplasia of Grebe type, an autosomal recessive disorder characterized by more severe limb shortening and dysmorphogenesis than the Hunter–Thompson type (Thomas *et al*, 1997). In these patients, the mutated CDMP-1 protein shows a dominant-negative effect by preventing the secretion of other BMP members (Thomas *et al*, 1997). It has been suggested that overexpression of BMP-4 mRNA in human lymphocytes is associated with fibrodysplasia ossificans progressiva, a heritable disorder of connective tissue characterized by postnatal formation of ectopic bone in muscular tissues (Shafritz *et al*, 1996).

Other BMP-deficient mice have also been created, although some of them died at stages too early in development to examine their skeletal phenotypes. BMP-7-deficient mice have skeletal patterning defects restricted to the rib cage, skull and hindlimbs (Dudley, Lyons and Robertson, 1995; Luo *et al*, 1995). Homozygous mutant mice carrying a targeted deletion of Gdf11 (also called BMP-11) exhibit anteriorly directed homeotic transformations throughout the axial skeleton and posterior displacement of the hindlimbs (McPherron, Lawler and Lee, 1999). The skeleton of BMP-6 null mice is indistinguishable from that of wild-type mice, suggesting that BMP-2 may functionally compensate in BMP-6-null mice (Solloway *et al*, 1998). BMP-4/7 double heterozygotes develop minor defects in the rib cage and the distal parts of limbs (Katagiri *et al*, 1998). These findings clearly indicate that BMPs are key regulators of the differentiation of osteoblasts and chondrocytes during skeletal development. However, it is still unclear whether BMPs are involved in bone and cartilage formation after birth. Interestingly, BMP-3-null mice have twice as much trabecular bone after birth as wild-type littermates, suggesting that BMP-3 is a negative determinant of bone density (Daluiski *et al*, 2001).

Role of BMPs in osteoblast differentiation in vitro

In order to examine the molecular mechanism of the 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 enhance the expression of ALP, parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor, type I collagen, and osteocalcin (Yamaguchi *et al*, 2000). Furthermore, BMPs stimulated the formation of mineralized bone-like nodules (Yamashita *et al*, 1996). BMPs also induced osteoblast differentiation in some other types of cells in culture. C3H10T1/2 clone 8 (10T1/2), a cell line established from a C3H mouse embryo, differentiates into myoblasts, adipocytes and chondrocytes in the presence 5-azacytidine (Taylor and Jones, 1979). We and others showed that BMP-2 and BMP-4 induce osteoblast differentiation of 10T1/2 cells (Katagiri *et al*, 1990; Ahrens *et al*, 1993; Wang *et al*, 1993). BMPs also stimulate osteoblast differentiation of other pluripotent cell lines (Yamaguchi *et al*, 1991; Rosen *et al*, 1994).

Bone morphogenetic proteins were originally identified as an activity that induces an ectopic bone formation in muscular tissue, suggesting that BMPs regulate the pathway of differentiation of myogenic cells. To examine this possibility, we used a mouse myoblast cell line, C2C12. We found that BMP-2 inhibited myogenic differentiation of C2C12 myoblasts, and converted their differentiation pathway into that of osteoblasts (Katagiri *et al*, 1994). TGF-β1 also inhibited myogenic differentiation of C2C12 cells, but failed to induce osteoblast differentiation of the cells (Katagiri *et al*, 1994). Similar effects of BMPs were observed in primary myoblasts and other myogenic cell lines in culture (Katagiri *et al*, 1994; unpublished observations). It has also been reported that the combination of BMP-2 gene transfer by adenoviruses and orthotopic muscle grafting in rats resulted in the successful ossification of almost the whole grafted muscle (Gonda *et al*, 2000). C2C12 cells are believed to have been derived from satellite cells of muscular tissue (Yaffe and Saxel, 1977; Blau, Chiu and Webster, 1983). Satellite cells are a potential source of regenerating myoblasts *in vivo*. These results suggest that satellite cells in muscular tissue are potential progenitors which can differentiate into osteoblasts in response to BMPs.

BMP receptors

Signaling by TGF-β superfamily members, including BMPs, is basically initiated upon their binding to

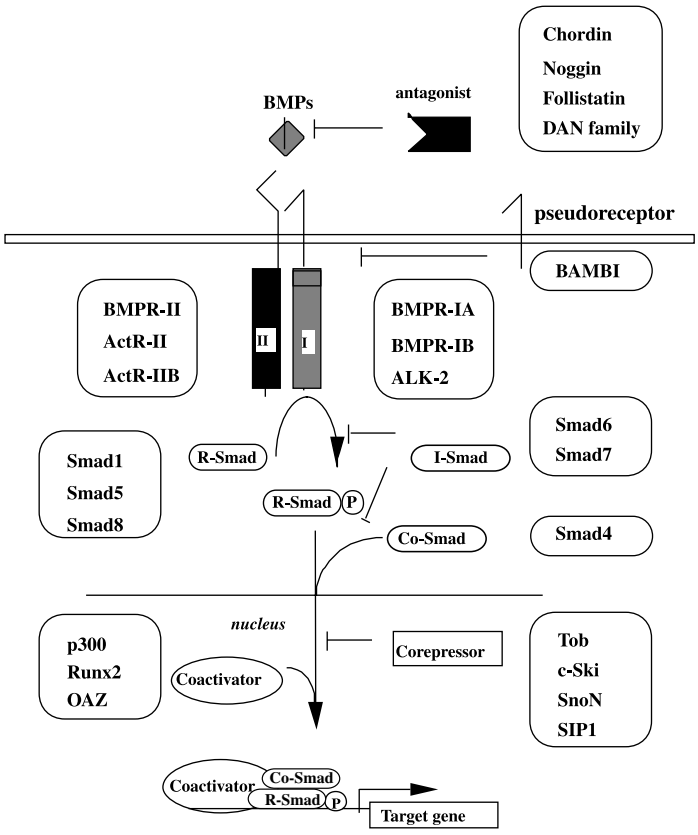

Figure 2 A schematic model for BMP signal transduction. Signaling of BMPs is initiated upon their binding to type I and II transmembrane receptors, which are serine/threonine kinases. The type I receptors phosphorylate Smad1/Smad5/Smad8 form a complex with Smad4. The complex moves into the nucleus and associates there with other DNA-binding proteins, and this large complex recognizes specific DNA motifs, and regulates the transcription of the target genes. This signaling pathway is regulated by a number of factors at multiple steps

the transmembrane receptors (Figure 2). Two types of the transmembrane serine/threonine kinase receptors, types I and II, are required for the signal transduction. The kinase activity of the ligand-bound type II receptor phosphorylates the GS domain of the type I receptor kinase. Substitution mutations of the type I receptor activated the downstream signal transduction without ligand-binding or phosphorylation by the type II receptor (Wieser, Wrana and Massague, 1995). Overexpression of the constitutively active BMP type I receptors, such as BMPR-IA, BMPR-IB and ALK-2, induced osteoblast differentiation in some types of cells (Akiyama *et al*, 1997; Chen *et al*, 1998; Fujii *et al*, 1999; Aoki *et al*, 2001). In contrast, when kinase domain-truncated BMP type I receptors were overexpressed in progenitor cells, the cells failed to differentiate into osteoblasts even in the presence of BMPs (Namiki *et al*, 1997; Chen *et al*, 1998). BMPR-IA is also involved in adipogenic differentiation of calvaria-derived cells (Chen *et al*, 1998). The binding of BMPs to receptors is regulated at multiple steps (Figure 2). The BMP type II receptors increase the ligand binding affinity of the type I receptors (Rosenzweig *et al*, 1995; Beppu *et al*, 1997). BAMBI, a pseudoreceptor of the TGF-β family, stably associates with type I receptors and inhibits BMP-, TGF-β- and activin- induced signals by preventing the formation of receptor complexes. (Onichtchouk *et al*, 1999). Several secreted proteins such as chordin, noggin, follistatin and DAN family members bind to BMPs and act as antagonists that inhibit the binding of the BMPs to receptors (Piccolo *et al*, 1996, 1999; Zimmerman, De Jesus-Escobar and Harland, 1996; Hsu *et al*, 1998; Iemura *et al*, 1998). Defects in joint development are observed in noggin-deficient mice (Brunet *et al*, 1998). BMP-1 acts as a protease that releases the carboxy-terminal propeptide from type I collagen (Kessler *et al*, 1996). Interestingly, a Xenopus homolog of BMP-1 releases active BMPs from the chordin–BMP complex by cleaving chordin (Piccolo *et al*, 1997).

Role of Smads in osteoblast differentiation

Smad transcription factors are substrates of the activated type I receptor kinases in the cytoplasm. The phosphorylated Smad proteins move into the nucleus, bind to the regulatory regions of target genes, and regulate their transcription. Thus, Smad proteins are key molecules in the transduction of signals from the cell membrane to the nucleus (Sakou, 1998; Miyazono, 1999; Massague, 2000; Massague and Chen, 2000; Wrana, 2000; Shi, 2001). So far nine Smad proteins have been identified in vertebrates. They are classified into three subgroups, R-Smad, Co-Smad and I-Smad, according to their structure and function. The R-Smads consist of Smad1, Smad2, Smad3, Smad5 and Smad8. They are directly phosphorylated by the type I receptors at the carboxy terminal SSXS motif (Kretzschmar *et al*, 1997). BMP type I receptors phosphorylate Smad1, Smad5 and Smad8, while TGF-β and activin type I receptors phosphorylate Smad2 and Smad3.

Overexpression of Smad1, Smad5 or Smad8 induces ALP activity and osteocalcin production in C2C12 and 10T1/2 cells (Yamamoto *et al*, 1997; Nishimura *et al*, 1998; Fujii *et al*, 1999; Kawai *et al*, 2000). Smad4 is one of the Co-Smads, which cooperate with all R-Smads. In contrast, both Smad6 and Smad7 inhibit signal transduction of the TGF-β superfamily members, so they are known as I-Smads (I indicates 'inhibitory'). I-Smads appear to be involved in a negative-feedback loop of the TGF-β superfamily signaling, because the expression of I-Smad mRNAs is rapidly induced by BMPs and TGF-βs. Signals other than Smad-mediated ones are also activated by the BMP type I receptors (Lou *et al*, 2000; Gallea *et al*, 2001). Therefore, Smad signals are regulated positively and negatively not only by Smads but also by transcriptional activators and/or repressors (Figure 2). Recently, Yoshida *et al* (2000) reported that tob-null mice have a greater bone mass, and their orthotopic bone formation is elevated relative to that in normal mice in response to BMP-2. They also showed that tob protein negatively regulates osteoblast proliferation and differentiation by suppressing the activity of R-Smads. BMP-2 and leukemia inhibitory factor synergistically stimulated astrocyte differentiation through the formation of a complex between Smad1 and STAT3, bridged by p300 protein (Nakashima *et al*, 1999). Thus, Smads appear to regulate the target gene expression through interaction with other transcription regulators.

Role of Runx2 and Osterix in osteoblast differentiation

The establishment of cbfa1-null mice clearly indicated that this transcription factor is essential for osteoblast differentiation, because, the mutant mice have no bone tissue or osteoblasts (Komori *et al*, 1997; Otto *et al*, 1997). Cbfa1/pebp2aA/AML3/osf-2 is a mammalian homolog of the Drosophila runt, and is now called Runx2. Moreover, Runx2 has also been identified as a gene responsible for cleidocranial dysplasia (CCD), an autosomal-dominant disease with abnormalities in bones formed by intramembranous ossification (Lee *et al*, 1997; Mundlos *et al*, 1997). The null mutation of Runx2 severely affects osteoblast differentiation but causes no abnormality in the patterning of the skeleton (Komori *et al*, 1997; Otto *et al*, 1997). Osteoblasts express high levels of Runx2 *in vivo* and *in vitro*. Runx2-deficient mice lack hypertrophic chondrocytes, suggesting that Runx2 also regulates chondrocyte differentiation (Komori *et al*, 1997). However, recent studies have revealed the complex role of Runx2 in osteoblast and chondrocyte differentiation. Overexpression of Runx2 in some non-osteoblastic cells induced the expression of osteoblast-related genes (Ducy *et al*, 1997; Harada *et al*, 1999). In contrast, Runx2 overexpression in osteoblasts *in vitro* suppressed the expression of type I collagen (Tsuji, Ito and Noda, 1998). Transgenic mice overexpressing either a dominant-negative or wild-type form of Runx2 in osteoblasts exhibited osteopenia (Ducy *et al*, 1999; Liu *et al*, 2001). Runx2 overexpression in chondrocytes *in vivo* caused acceleration of endochondral ossification in mice because of precocious chondrocyte maturation (Takeda *et al*, 2001; Ueta *et al*, 2001). In contrast, overexpression of dominant-negative Runx2 in chondrocytes *in vivo* suppressed their maturation and delayed endochondral ossification (Ueta *et al*, 2001). Furthermore, continuous expression of wild-type Runx2 in non-hypertrophic chondrocytes partially induced mineralization of cartilage in Runx2-null mice (Takeda *et al*, 2001). However, transdifferentiation from chondrocytes into osteoblasts was not observed in these mice (Takeda *et al*, 2001). Thus, Runx2 plays intricate roles in osteoblast and chondrocyte development.

Bone morphogenetic proteins up-regulate Runx2 mRNA expression *in vitro* (Ducy *et al*, 1997; Tsuji *et al*, 1998). Hanai *et al* (1999) showed that R-Smads interact with Runx1/Runx2/Runx3. Zhang, Yasui and Ito (2000) also reported that a truncated Runx2 identified in a CCD patient failed to interact with Smads. Runx2 cooperated with Smads to induce osteoblast differentiation of C2C12 cells (Lee *et al*, 2000; Zhang *et al*, 2000). These lines of evidence suggest that Runx2 interacts tightly with BMP signaling through Smads in osteoblast differentiation. Further studies will be necessary to reveal the precise relationship between Runx2 and transcription factors, including Smads, in the induction of osteoblast differentiation. Elucidation of the regulatory mechanism of osteoblast differentiation will provide a new approach to the treatment of oral diseases.

Recently, Nakashima *et al* (2002) identified a novel zinc finger-containing transcription factor, named Osterix, from C2C12 cells treated with BMP-2. In Osterix-null mice, no bone formation occurred although Runx2 was expressed. Interestingly, however, Osterix was not expressed in Runx2-null mice. These results suggest that Osterix acts downstream of Runx2 during bone development.

Regulation of osteoclast differentiation

Osteoblasts regulate osteoclastogenesis

Development of osteoclasts proceeds within the local microenvironment of bone. A coculture system of mouse osteoblasts/stromal cells and hemopoietic cells was developed to investigate the regulatory mechanisms of osteoclast differentiation (Takahashi *et al*, 1988; Suda, Takahashi and Martin, 1992). Osteoclast-like multinucleated cells are formed in the cocultures in response to various osteotropic factors including 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], PTH, prostaglandin E_2 (PGE_2) and interleukin 11 (IL-11). Those multinucleated cells formed in the coculture expressed major characteristics of osteoclasts such as tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts), calcitonin receptors, $p60^{c\text{-}src}$, vitronectin receptors ($\alpha v\beta 3$), and the ability to form resorption pits on bone and dentine slices. Some mouse stromal cell lines such as MC3T3-G2/PA6 and ST2 resemble calvarial osteoblasts and support osteoclastogenesis in cocultures with mouse spleen cells (Udagawa *et al*, 1989). Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is required to induce osteoclastogenesis. The target cells of osteotropic factors for inducing osteoclast

formation in the cocultures are osteoblasts/stromal cells (Udagawa *et al*, 1995; Liu *et al*, 1998; Takeda *et al*, 1999; Sakuma *et al*, 2000). Therefore, we have proposed that osteoblasts/stromal cells induce osteoclast differentiation factor (ODF) as a membrane-associated cytokine in response to various osteotropic factors (Suda *et al*, 1992). Osteoclast progenitors recognize ODF through cell-to-cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts.

A method for obtaining highly purified osteoclasts from cocultures was established to investigate the role of osteoblasts/stromal cells in osteoclast function (Jimi *et al*, 1996; Suda *et al*, 1997). Purified osteoclasts cultured on dentine slices failed to form resorption pits. The resorptive capability of the purified osteoclasts was restored when osteoblasts/stromal cells were added to the purified osteoclasts. Cell-to-cell contact between osteoblasts/stromal cells and osteoclasts was required for inducing the pit-forming activity of osteoclasts (Jimi *et al,* 1996). Thus, osteoblasts/stromal cells play essential roles in inducing osteoclast function.

Characteristics of osteoclast progenitors

Several lines of evidence indicate that osteoclast progenitors are hemopoietic cells of the monocyte/macrophage lineage. Osteopetrotic op/op mice cannot produce functionally active macrophage colony-stimulating factor (M-CSF, also called CSF-1) because of an insertion of an extra thymidine in the coding region of the M-CSF gene (Yoshida *et al*, 1990). Experiments using the op/op mouse model have established that M-CSF produced by osteoblasts/stromal cells is a crucial factor for osteoclast formation. Administration of M-CSF to op/op mice restored impaired bone resorption (Felix, Cecchini and Fleisch, 1990; Kodama *et al*, 1991). Osteoclast progenitors in the spleen obtained from op/op mice differentiated into osteoclasts in cocultures with normal osteoblasts (Takahashi *et al*, 1991). However, calvarial osteoblasts prepared from op/op mice failed to support osteoclast development in cocultures with normal spleen cells, and the addition of M-CSF to the cocultures induced osteoclast formation in response to $1,25(OH)_2D_3$. These findings indicate that M-CSF produced by osteoblasts/stromal cells plays an essential role in osteoclast development. Mouse peripheral blood mononuclear cells and alveolar macrophages differentiated into osteoclasts in coculture with ST2 cells, a supportive stromal cell line (Udagawa *et al*, 1990). The results of disruption of the PU.1 gene in mice also supported the monocyte/macrophage origin of osteoclasts (Tondravi *et al*, 1997). PU.1 is a myeloid- and B-cell-specific transcription factor, and PU.1(–/–) mice were found to be osteopetrotic. The development of both osteoclasts and macrophages was arrested in PU.1(–/–) mice, suggesting that this transcription factor regulates the initial stage of myeloid differentiation.

Discovery of new TNF receptor-ligand family members involved in osteoclastogenesis

The recent discovery of new members of the TNF receptor-ligand family has clarified the precise mechanism by which osteoblasts/stromal cells regulate osteoclast differentiation and function. Simonet *et al* (1997) cloned a new member of the tumor necrosis factor (TNF) receptor family, termed osteoprotegerin (OPG), in an expressed sequence tag cDNA project. OPG lacks a transmembrane domain and represents a secreted TNF receptor member. Hepatic expression of OPG in transgenic mice results in osteopetrosis. Tsuda *et al* (1997) independently isolated a novel protein termed osteoclastogenesis inhibitory factor (OCIF) from the conditioned medium of human fibroblast cultures. The sequence of the cDNA for OCIF was identical to that of the cDNA for OPG. OPG strongly inhibited osteoclast formation induced by $1,25(OH)_2D_3$, PTH, PGE_2 or IL-11 in cocultures. Using OPG as a probe, a cDNA with an open reading frame encoding 316 amino acid residues was cloned from an expression library of ST2 cells (Yasuda *et al*, 1998). The OPG-binding molecule was a type II transmembrane protein of the TNF ligand family, and its expression in osteoblasts/stromal cells was up-regulated by osteotropic factors including $1,25(OH)_2D_3$, PGE_2, PTH and IL-11. A soluble form of this OPG-binding molecule together with M-CSF induced osteoclast formation from spleen cells in the absence of osteoblasts/stromal cells, and this osteoclast formation was completely inhibited by adding OPG. Thus, the OPG-binding molecule satisfied the major criteria of ODF, and therefore this molecule was renamed ODF (Yasuda *et al*, 1998). Lacey *et al* (1998) also cloned a ligand for OPG (OPGL), and it was found that OPGL was identical to ODF. Molecular cloning of ODF/OPGL demonstrated that it is identical to TRANCE (TNF-related activation-induced cytokine) (Wong *et al*, 1997) and receptor activator of nuclear factor κB ligand (RANKL) (Anderson *et al*, 1997), which had been independently identified by other research groups. TRANCE was cloned during a search for apoptosis-regulatory genes in mouse T cell hybridomas. TRANCE induced activation of c-Jun N-terminal kinase (JNK) in T lymphocytes and inhibited apoptosis of mouse and human dendritic cells (Wong *et al*, 1997). A new member of the TNF receptor family, termed 'RANK', was cloned from a cDNA library of human dendritic cells (Anderson *et al*, 1997). The mouse homolog was also isolated from a fetal mouse liver cDNA library. The mouse RANK cDNA encodes a type I transmembrane protein of 625 amino acid residues. Thus, ODF, OPGL, TRANCE and RANKL are different names for the same molecule, a protein which is important for the development and function of T cells, dendritic cells and osteoclasts. RANK is the transmembrane signaling receptor for ODF/OPGL/TRANCE/RANKL. OPG/OCIF is a soluble decoy receptor for ODF/OPGL/TRANCE/RANKL. The terms 'RANKL', 'RANK' and 'OPG' are used in this article in accordance with the guidelines of The American Society for Bone and Mineral Research President's Committee on Nomenclature (2000). RANKL stimulates the pit-forming activity of mature osteoclasts (Burgess *et al*, 1999; Jimi *et al*, 1999a). Human osteoclasts are also formed in cultures of human peripheral

blood mononuclear cells in the presence of RANKL and human M-CSF (Matsuzaki *et al*, 1998).

RANKL–RANK interaction in osteoclastogenesis

The expression of RANKL in osteoblasts/stromal cells is up-regulated by osteotropic hormones and factors such as $1,25(OH)_2D_3$, PTH, PGE_2 and IL-11. Compounds that elevate intracellular calcium, such as ionomycin, cyclopiazonic acid and thapsigargin, also induced osteoclast formation in mouse cocultures of bone marrow cells and primary osteoblasts (Takami *et al*, 1997) (Figure 3). Similarly, high calcium concentrations in the culture medium induced osteoclast formation in the cocultures. Treatment of primary osteoblasts with these compounds or the medium containing high levels of calcium stimulated the expression RANKL and OPG mRNAs (Takami *et al*, 2000). These results suggest that independent signals mediated by vitamin D receptors (VDR), cAMP, gp130 and intracellular calcium induce expression of RANKL in osteoblasts/stromal cells (Figure 3).

Receptor activator of NF-κB ligand knockout(–/–) mice exhibit typical osteopetrosis, with total occlusion of the bone marrow space within endosteal bone (Kong *et al*, 1999). RANKL(–/–) mice lack osteoclasts but have normal osteoclast progenitors that can differentiate into functionally active osteoclasts when cocultured with normal osteoblasts/stromal cells. Like RANKL-deficient mice, RANK(–/–) mice are characterized by severe osteopetrosis (Dougall *et al*, 1999). The osteopetrosis observed in RANK(–/–) mice but not RANKL(–/–) mice is rescued by transplantation of normal bone marrow cells, indicating that RANK(–/–) mice have an intrinsic defect in osteoclast lineage cells. These data indicate that RANK is the intrinsic cell surface determinant that mediates the effects of RANKL on bone resorption. A gene mapping study showed that the gene responsible for familial expansile osteolysis and familial Paget's disease of bone mapped to the gene encoding RANK (Hughes *et al*, 2000). This finding confirms that RANK is involved in osteoclast differentiation and activation in humans as well.

Activation of NF-κB and JNK through the RANK-mediated signaling system appears to be involved in the differentiation and activation of osteoclasts. The cytoplasmic tail of RANK interacts with TNF-associated factor 1 (TRAF1), TRAF2, TRAF3, TRAF5 and TRAF6 (Darnay *et al*, 1998; Galibert *et al*, 1998; Wong *et al*, 1998; Darnay *et al*, 1999; Kim *et al*, 1999). TRAF6-mediated signals appear to be important for osteoclast differentiation and function, because TRAF6(–/–) mice develop osteopetrosis with defects in bone resorption and tooth eruption (Lomaga *et al*, 1999; Naito *et al*, 1999). Mice deficient in both the p50 and p52 subunits of NF-κB develop severe osteopetrosis (Franzoso *et al*, 1997; Iotsova *et al*, 1997). The osteopetrotic phenotype was rescued by bone marrow transplantation, indicating that the osteoclast progenitors are inactive in the double-knockout mice. RANKL-induced activation of NF-κB in osteoclast progenitors seems to play a crucial role in osteoclast differentiation. Mice lacking c-Fos also develop osteopetrosis because of an early block of differentiation in the osteoclast lineage (Wang *et al*, 1992; Grigoriadis *et al*, 1994). The dimeric transcription factor activator protein-1 (AP-1) is composed of mainly Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) and Jun proteins (c-Jun, JunB and JunD). These results suggest that AP-1 appears to act downstream of RANK-mediated signals.

Role of inflammatory cytokines in osteoclastogenesis

Since the discovery of the RANKL–RANK signaling system, RANKL has been regarded as the sole factor responsible for inducing osteoclast differentiation. However, recent findings indicate that inflammatory cytokines and LPS are directly involved in osteoclast differentiation and function (Figure 4).

Interleukin-1 directly stimulates osteoclast function through the IL-1 type 1 receptor expressed by osteoclasts (Jimi *et al*, 1999b). The pit-forming activity of

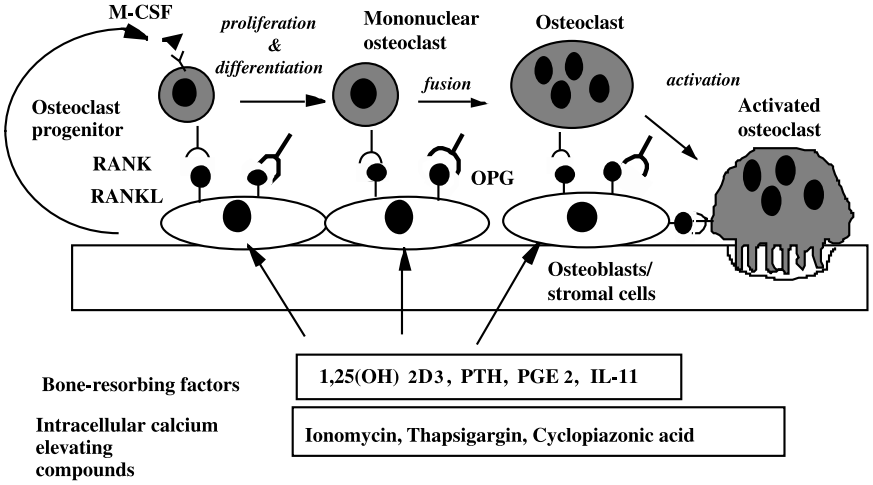

Figure 3 A schematic representation of osteoclast differentiation and function supported by osteoblasts/stromal cells. Osteotropic factors such as 1,25 $(OH)_2D_3$, PTH, PGE_2 and IL-11 stimulate the expression of RANKL as a membrane associated factor in osteoblasts/stromal cells. Compounds that elevate intracellular calcium, such as ionomycin, cyclopiazonic acid and thapsigargin, also induce RANKL expression in osteoblasts/stromal cells. Osteoclast progenitors of the monocyte-macrophage lineage recognize RANKL expressed by osteoblasts/stromal cells through cell-to-cell interaction, and differentiate into osteoclasts. M-CSF produced by osteoblasts/stromal cells is another essential factor for osteoclast differentiation. RANKL expressed by osteoblasts/stromal cells also stimulates osteoclast function through cell-to-cell interaction

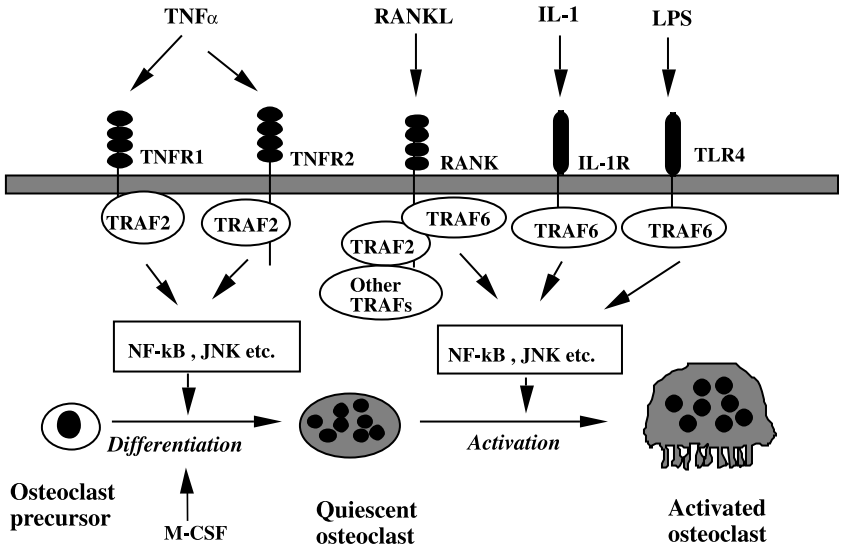

Figure 4 Schematic representation of ligand–receptor systems in osteoclast differentiation and function regulated by TNFα, RANKL, IL-1 and LPS. TNFα and RANKL independently stimulate osteoclast differentiation via TNFR1 and TNFR2, and RANK expressed by osteoclast precursors, respectively. M-CSF is a common factor required for TNFα- and RANKL-induced osteoclast differentiation. TRAF2 and other TRAFs may transduce signals for the differentiation of osteoclasts. The activation of osteoclasts is induced by RANKL, IL-1 and LPS through RANK, type 1 IL-1 receptor and TLR4, respectively. TRAF6 appears to act as a common signal transducer in osteoclast activation induced by RANK, IL-1 and LPS. Signal transduction cascades such as NF-κB and JNK activation may be involved in the differentiation and activation of osteoclasts

osteoclasts induced by IL-1 was completely inhibited by adding IL-1 receptor antagonist (IL-1ra) but not by OPG. LPS is a cell component of Gram-negative bacteria that causes inflammatory bone loss. Recent studies identified mouse toll-like receptor 4 (TLR4) as the receptor for LPS (Poltorak *et al*, 1998; Hoshino *et al*, 1999; Qureshi *et al*, 1999). The cytoplasmic signaling cascade of TLR4 is similar to that of IL-1 receptors. Both receptors have been shown to use TRAF6 as a common signaling molecule. To examine the effect of LPS on the survival and fusion of osteoclasts, mononuclear osteoclasts (preosteoclasts, pOCs) were collected from a mouse coculture system and cultured in the presence or absence of LPS (Suda *et al*, 2001). Most pOCs died within 24 h in the absence of any stimulus. LPS as well as RANKL supported the survival of pOCs, and induced their fusion to form multinucleated osteoclasts. LPS-induced osteoclast formation in pOC cultures was observed even in the presence of OPG and IL-1 receptor antagonists. LPS induced pit-forming activity of pOCs in the presence of M-CSF. These findings suggest that LPS as well as IL-1 stimulates the survival and fusion of pOCs.

Recent studies have shown that TNFα directly stimulates the differentiation of osteoclast progenitors into osteoclasts in the presence of M-CSF (Azuma *et al*, 2000; Kobayashi *et al*, 2000). When mouse bone marrow cells were cultured with M-CSF, M-CSF-dependent bone marrow macrophages appeared within 3 days. Not only RANKL but also TNFα stimulates the differentiation of these macrophages into osteoclasts in the presence M-CSF. Osteoclast formation induced by TNFα was inhibited by the addition of the respective antibodies against TNF receptor type I (TNFRI, p55) and TNF receptor type II (TNFRII, p75), but not by the addition of OPG. These results demonstrate that TNFα stimulates osteoclast differentiation through a mechanism independent of the RANKL–RANK system. It was also reported that when osteotropic factors such as $1,25(OH)_2D_3$, PTHrP and IL-1 were administered to RANK(–/–) mice, neither TRAP-positive cell formation nor hypercalcemia was induced (Li *et al*, 2000). In contrast, administration of TNFα to RANK(–/–) mice induced TRAP-positive cells near the site of injection, although the number of TRAP-positive cells induced by TNFα was not large. This suggests that TNFα induces osteoclast differentiation in the absence of RANK-mediated signals *in vivo*. Lam *et al* (2000) also reported that a small amount of RANKL strongly enhanced osteoclast differentiation in a pure population of murine precursors in the presence of TNFα. These results suggest that RANKL-induced signals cross-communicate with TNFα-induced ones in the target cells (Figure 4). Thus, these cytokines and LPS play important roles in osteoclastic bone resorption induced by inflammatory diseases including periodontitis. Further studies will be necessary to elucidate the regulatory mechanisms of osteoclastic bone resorption induced by inflammatory cytokines and LPS.

Role of TGF-β super family members and interferon-γ in osteoclastogenesis

Bone is a major storage site for TGF-β super family members such as TGF-β and BMPs, and osteoclastic bone resorption releases these cytokines. TGF-β has been shown to enhance osteoclast differentiation in hematopoietic cells stimulated with RANKL and M-CSF (Sells Galvin *et al*, 1999; Quinn *et al*, 2001). Fuller, Bayley and Chambers (2000a) reported that activin A also powerfully synergized with RANKL for induction of osteoclasts from their progenitors. Moreover, osteoclast formation induced by RANKL was completely abolished by soluble activin receptor type IIA or soluble TGF-β receptor II, suggesting that activin A and TGF-β are essential factors for osteoclastogenesis (Fuller *et al*, 2000a; b). We also showed that BMP-2 strikingly stimulated osteoclast differentiation in the presence of RANKL and M-CSF (Itoh *et al*, 2001). OPG completely inhibited osteoclast differentiation induced by RANKL and BMP-2. A soluble form of

BMP receptor type-IA also inhibited osteoclast formation in the presence of RANKL (Itoh *et al*, 2001). We found that BMP receptor type IA mRNA was expressed on not only osteoclast progenitors but also mature osteoclasts, and that BMP-2 enhanced the survival of purified osteoclasts in the presence of RANKL but not M-CSF (Itoh *et al*, 2001). Smad1 and Smad5 are involved in the BMP signals, whereas Smad2 and Smad3 in the TGF-β signals in the target cells. However, both BMP and TGF-β showed similar effects on osteoclast progenitors. This suggests that signaling pathways other than Smad-mediated pathways are involved in enhancement of RANKL-induced osteoclast differentiation by TGF-β super family members. Further studies are necessary to elucidate the molecular mechanism of the crosstalk between BMPs and RANKL in osteoclastogenesis.

Bone resorption is regulated by the immune system, where T-cell expression of RANKL may contribute to pathological conditions, such as periodontitis and autoimmune arthritis. Activated T cells also produce interferon (IFN)-γ, which strongly suppresses osteoclastogenesis by interfering with the RANKL–RANK signaling pathway. Takayanagi *et al* (2000) reported that IFN-γ induced rapid degradation of TRAF6, which resulted in strong inhibition of the RANKL-induced activation of NF-κB and JNK. This inhibition of osteoclastogenesis was rescued by overexpressing TRAF6 in precursor cells, suggesting that TRAF6 is the target critical for the IFN-γ action. These results indicate that there is crosstalk between the TNF and IFN families of cytokines, through which IFN-γ provides a negative link between T-cell activation and bone resorption.

Conclusion

Bone morphogenetic proteins play critical roles in osteoblast differentiation. Smad-mediated signals are essential in BMP-induced osteoblast differentiation. Runx2 and Osterix are transcription factors required for osteoblast differentiation and bone formation. RANKL–RANK interaction is absolutely necessary for osteoclast differentiation. LPS and some inflammatory cytokines such as TNFα and IL-1 are directly involved in osteoclast differentiation and function through a mechanism independent of RANKL–RANK interaction. TGF-β super family members and IFN-γ are also important regulators in osteoclastogenesis. Further studies on the regulatory mechanisms of osteoblast and osteoclast differentiation will provide novel approaches for the treatment of bone and oral diseases.

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