Molecular biology of bone remodelling

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Summary

Bone remodelling is an active and dynamic process that relies on the correct balance between bone resorption by osteoclasts and bone deposition by osteoblasts. Moreover, these two functions must be tightly coupled not only quantitatively, but also in time and space. When the coupling is lost, the correct bone mass could be compromised, leading to several skeletal pathologies. Indeed, bone loss and osteoporosis are the result of an increased osteoclast function and/or a reduced osteoblast activity. In contrast, other pathologies are related to osteoclast failure to resorbe bone, such as osteopetrosis, a rare genetic disorder characterized by an increased bone mass and also linked to an impairment of bone marrow function. Starting from these assumptions, it is necessary to more deeply understand the molecular mechanisms regulating bone cell functions. Indeed, recent studies evidenced a complex interplay between the immune and skeletal systems, which share several regulatory molecules including cytokines, receptors and transcription factors. These data allowed to more deeply understand the mechanisms underlying bone mass regulation and could open new avenue to identify target molecules for alterantive therapies more efficacious against bone diseases.

KEY WORDS: osteoclast, osteoblast, bone remodelling, osteoimmunology.

The bone remodelling process

Bone is a dynamic tissue, subjected to a continuous renewing during the life of each individual by the process of bone remodelling (1, 2). This physiological process is necessary:

– to allow the substitution of primary bone, the infantile bone, with secondary bone which is more mechanically competent;
– to remove ischemic or microfractured bone;
– to guarantee a correct calcium homeostasis.

Bone remodelling relies on the correct function of two principal cells of the bone tissue: the osteoclasts, multinucleated cells that destroy the bone matrix, and the osteoblasts, having osteogenic functions. The osteocytes, another important cell type arising from the osteoblasts, are also involved in the remodelling process as they have a mechano-sensorial function (3).

A correct balance between bone resorption and osteogenic functions is mandatory to maintain a constant bone mass (1, 2).

As summarized in Figure 1, bone remodelling is accomplished according to the following phases.

Activation phase. Different inputs, such as a micro-fracture, an alteration of mechanical loading sensed by the osteocytes or some factors released in the bone microenvironment, including insulin growth factor-I (IGFI), tumour necrosis factor-α (TNF-α), parathyroid hormone (PTH) and interleukin-6 (IL-6), activate the lining cells which are quiescent osteoblasts. As a consequence, lining cells, increase their own surface expression of RANKL (Receptor Activator of Nuclear κB Ligand), which in turn interacts with its receptor RANK (Receptor Activator of Nuclear κB), expressed by pre-osteoclasts. RANKL/ RANK interaction triggers pre-osteoclasts fusion and differentiation toward multinucleated osteoclasts.

Resorption phase. Once differentiated, osteoclasts polarize, adhere to the bone surface and begin to dissolve bone. This function requires two steps: i) acidification of the bone matrix to dissolve the inorganic component, and ii) release of lysosomial enzymes, such as cathepins K, and of MMP9, both in charge for the degradation of the organic component of bone. Once accomplished their function, osteoclasts undergo to apoptosis. This is a physiological consequence needed to avoid an excessive bone resorption.

Reverse phase. The reverse cells, whose role has not been yet completely clarified, perform this phase. Indeed, it is known that they are macrophage-like cells with a likely function of removal of debris produced during matrix degradation.

Formation phase. Bone matrix resorption leads to the release of several growth factors herein stored, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and transforming growth factor β (TGF β), which are likely responsible for the recruitment of the osteoblasts in the reabsorbed area. Once recruited, osteoblasts produce the new bone matrix, initially not calcified (osteoid) and then they promote its mineralization, thus completing the bone remodelling process.

Unbalance between the resorption and formation phases mirror an incorrect bone remodelling, which in turn affects the bone mass, eventually leading to a pathological condition.

The bone remodelling players

To more deeply understand the causes of an altered bone remodelling it is necessary to know the mechanisms underlying the biology and function of bone cells. As already mentioned, two principal bone cells actively attend the bone remodelling, that is the osteoblasts and the osteoclasts, and an overview of their regulation and function will be done.

Regulation and function of osteoblasts

Osteoblastogenesis

Osteoblasts arise from mesenchymal stem cells (MSCs), which are pluripotent cells that following a specific program of gene

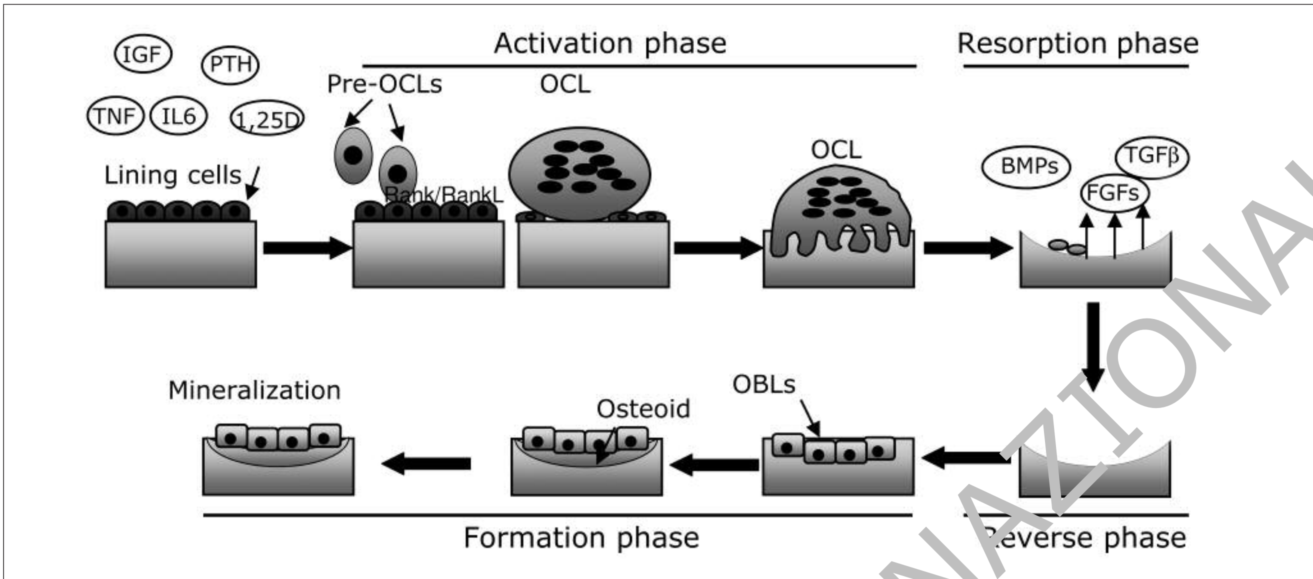

Figure 1 - Schematic representation of the bone remodelling process. Bone remodelling starts when different inputs led to activation of lining cells, which increase surface expression of RANKL. RANKL interacts with its receptor RANK (Receptor Activator of Nuclear κB) thus triggering osteoclast differentiation (Activation phase). Osteoclasts resorbe bone (Resorption Phase) thus allowing the release of factors usually stored in the bone matrix (BMPs, TGFβ, FGFs) that recruit osteoblasts in the reabsorbed area. Once recruited, osteoblasts produce the new bone matrix, and promote its mineralization (Formation phase), thus completing the bone remodelling process (Pre-OCLs = pre-osteoclasts; OCL = osteoclast; OBLs = osteoblasts).

expression may give rise to different tissue specific cells including osteoblasts, chondrocytes, fibroblasts, myocytes and adipocytes (4, 5).

The initial step of osteoblastogenesis is the commitment of MSCs towards an osteo/chondro-progenitor (Figure 2). As described in more detail later, the Wingless-int (Wnt) pathway and the BMPs play a key role in these early events. Indeed a recent report (6) showed that Wnt10b not only shifts the commitment towards an osteo/chondro progenitor, but also inhibits preadipocyte commitment (Figure 2). This is due to the suppression of the adipogenic transcription factors CCAAT enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) along with to an induction of transcription factors Runt-related transcription factor 2 (Runx2), distal-less homeobox 5 (Dlx5), and Osterix (Osx), the latter downstream of Runx2 (7). Conversely, high levels of Wnt signaling with the presence of Runx2 promote osteoblastogenesis at the expense of chondrocyte differentiation (8). Committed pre-osteoblasts are identifiable as they express Alkaline Phosphatase (ALP), one of the earliest markers of osteoblast phenotype. As the pre-osteoblasts cease to proliferate, a key signalling event occurs for development of the large cuboidal differentiated osteoblasts. The active osteoblast is highly enriched in ALP and secrete bone matrix proteins such as collagen I and several non-collagenous proteins including osteocalcin, osteopontin, osteonectin and bone sialoprotein II (BSPII). As a rule, ALP and the type 1 parathyroid receptor (PTH1R) are early markers of osteoblast progenitors that increase as osteoblasts mature and deposit matrix, but decline again as osteoblasts become osteocytes, whereas osteocalcin is a late marker that is up-regulated only in post-proliferative mature osteoblasts associated with mineralized osteoid (9).

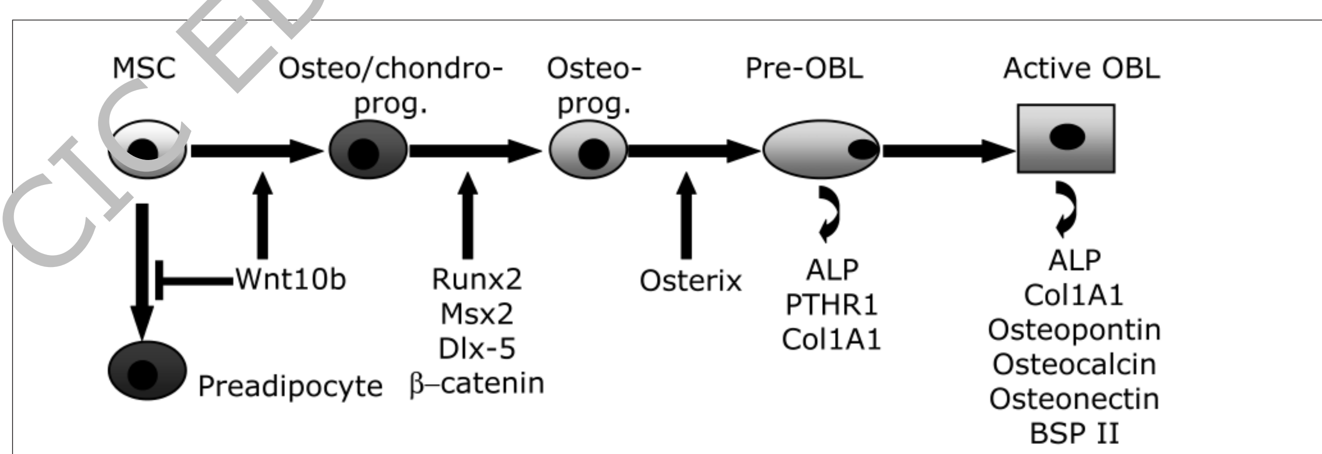

Figure 2 - Schematic picture of the osteoblastogenesis process. Osteoblasts (OBL) arise from mesenchymal stem cells (MSC) which under proper stimuli are committed toward an osteo/chondro-progenitor (osteo/chondro-prog.), followed by an osteoprogenitor cell (Osteo-prog.), a pre-osteoblast (pre-OBL) expressing alkaline phosphatase (ALP) and an active, mature osteoblast which secretes bone matrix proteins.

a) Osteoblast regulation

A correct osteoblastogenesis relies on the activation of a complex network of pathways whose alteration causes several skeletal pathologies. In the next paragraphs we will focus on some of the principal mechanisms of osteoblast regulation.

Runt-related transcription factor 2 (Runx2). This transcription factor plays a key role in skeletal development as it is a master gene for osteoblast differentiation, driving the early steps of mesenchymal commitment toward the pre-osteoblast phenotype (10-12). Indeed, in Runx2 null mice lack of osteoblast differentiation results in the absence of bone formation, and chondrocytes of cartilages templates fail to undergo hypertrophy, while overexpression of a dominant-negative form of Runx2 in osteoblasts inhibits bone formation (13). Interestingly, Runx2 overexpression also leads to osteopenia, thus indicating that this factor at inappropriately high levels can inhibit the process of osteoblast maturation (14). In humans, haploinsufficiency of Runx2 causes cleidocranial dysplasia (CCD), an autosomal-dominant disease with abnormalities in bones formed by intramembranous ossification (15, 16).
Among the molecules able to regulate Runx2, BMPs, TGF β, PTH and FGFs promote its activation, while the transcription factor Twist is a negative regulator (17).

Osterix (Osx). This factor is downstream of Runx2 and, like the latter, is necessary for skeletal formation (18). To accomplish this function Osx needs to interact with activated NFAT2 (19).

Wnt/β-catenin signalling. Recent reports evidenced a pivotal role of this pathway in bone biology (20, 21). Indeed, the great interest for Wnt signalling in bone field came after the discovery that loss and gain-of-function mutations in the Low-density lipoprotein receptor-related protein 5 (LRP5), a putative Wnt co-receptor, led to the osteoporosis-pseudoglioma syndrome (22) and to high bone mass (HBM) (23, 24) respectively in humans. LRP5 is a transmembrane receptor, which interacts with the frizzled receptor. Binding of Wnt to frizzled and LRP5/6 receptor complex triggers a signal involving the proteins Disheveled (Dvl), Axin and Frat-1, thus inhibiting the activity of the Glycogen synthase kinase 3β (GSK3β) (25). This inhibition prevents β-catenin phosphorylation. Indeed, hypophosporylated β-catenin is more stable, thus accumulating in the cytoplasm. Upon reaching a certain concentration level, β-catenin translocates to the nucleus where it interacts with the Tcf/Lef family of transcription factors to regulate the expression of Wnt target genes. In contrast, in the absence of Wnt, GSK3β phosphorylates β-catenin, thus targeting the protein to proteasome ubiquitination (26, 27).
Wnt signalling is subjected to a fine tune regulation by several factors. Among them, the members of the secreted frizzled-related protein (sFRP) family and Wnt inhibitory factor 1 (Wif-1). These molecules are soluble decoy frizzled receptors that prevent interactions between Wnt and frizzled. A second group of inhibitors includes dickkopf (Dkk) and sclerostin (Sost) proteins, which bind to LRP5/6 receptors. Moreover, interaction of the Dkk/LRP complex with kremen internalises the complex for degradation, thus reducing the availability of Wnt receptors (28).

Bone Morphogenetic Proteins (BMPs). Except for BMP-1, all these proteins belong to the TGF-β superfamily. Identification of skeletal abnormalities in animals and patients with mutations in the BMP genes has highlighted the role of these proteins in bone metabolism (29-31). *In vitro* studies demonstrated that treatment with BMPs enhances the expression of ALP, parathyroid hormone related peptide (PTHrP) receptor type I, collagen I and osteocalcin (32) and stimulated the formation of mineralized bone-like nodules (33).

b) Osteoblast function

As already described, the principal function of the osteoblasts is to synthesize the proteins of the bone matrix and to attend the process of calcification. Indeed, several evidence reported a crucial role of osteoblasts in osteoclast biology by expressing and/or secreting key molecules that in turn regulate osteoclastogenesis and bone resorption (1). This latter issue will be showed in detail in the next paragraphs.

Regulation and function of osteoclasts

Osteoclastogenesis

Osteoclasts, the cells devoted to resorb the bone matrix, arise from the monocyte/macrophage lineage (34). They are multinucleated cells (from four up to twenty nuclei) formed by the fusion of mononuclear precursors (35). Starting from totipotent heamatopoietic stem cells, the transcription factor PU.1, along with the macrophage colony stimulating factor (M-CSF) drive the commitment of a common progenitor for macrophages and osteoclasts. In particular, M-CSF stimulates proliferation of osteoclast precursors and upregulates RANK expression, while PU.1 positively regulates the transcription of c-Fms, the M-CSF receptor (36). With the appearance of c-Fms and RANK receptors, the precursors become fully committed to an osteoclast lineage. RANKL pathway is mandatory for osteoclast differentiation and function, although it is not the only player for a correct osteoclastogenesis, as described below.

a) Osteoclast regulation

RANKL pathway. RANKL is a type II membrane protein belonging to the TNF superfamily, while its receptor RANK is a type I membrane protein. Osteotropic hormones and factors such as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), PTH, prostaglandin E2 (PGE2) and IL-11 up-regulate the expression of RANKL on membrane surface of osteoblasts/stromal cells. RANKL interacts with its receptor RANK, located on the pre-osteoclast surface, which in turn activates the signalling by recruiting adaptor molecules belonging to the TNF-receptor associated factors (TRAF) family (Figure 3A). Indeed, RANK cytoplasmic tail contains three binding sites for TRAF6 (37-39). This interaction is mandatory for osteoclast differentiation, as TRAF6 knock out mice develop osteopetrosis (40, 41). The binding of TRAF6 to RANK induces the trimerization of TRAF6, leading to the activation of the transcription factor nuclear factor kappaB (NF-κB) and of the mitogen-activated kinases (MAPKs) (42) (Figure 3A).
NF-κB includes a family of dimeric transcription factors, which reside in the cytoplasm in non-stimulated conditions. However, they enter nucleus upon cell stimulation of various factors, including RANKL (43, 44) and regulate transcription of several genes. Among them, it has been demonstrated that NFkB upregulates the expression of another key molecule of osteoclast differentiation, that is the nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) transcription factor (45, 46). This initial induction requires the interaction of NF-κB with NFATc2, which is recruited to the NFATc1 promoter independently of RANKL stimulation (47) (Figure 3A).
Another crucial step for osteoclasts differentiation is the recruitment of the transcription factor complex AP-1, which is composed of the c-Fos, c-Jun and ATF proteins. In particular, c-Fos is specifically induced by RANK and is critical for osteo-

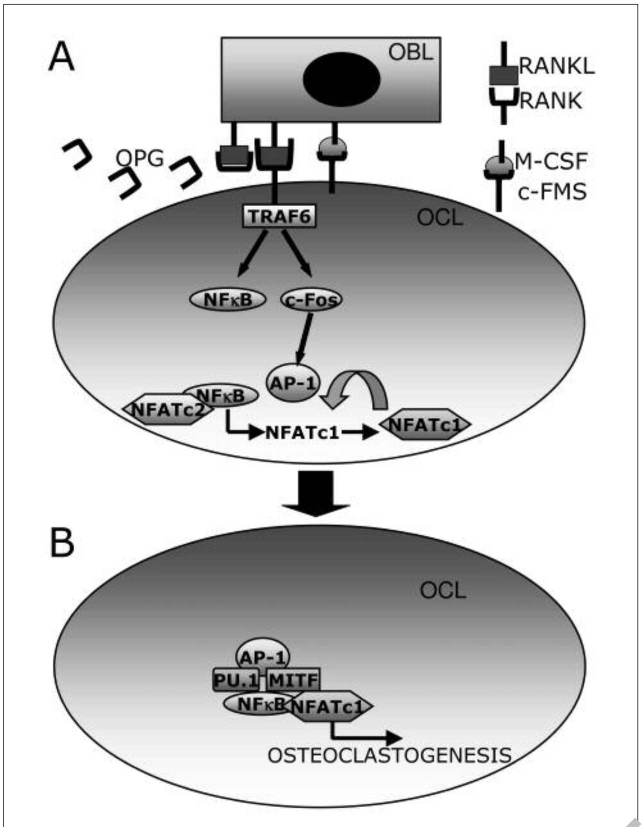

Figure 3 - Schematization of the RANKL/RANK pathway. (A) RANKL expressed on the membrane surface of osteoblasts (OBL) interacts with RANK, expressed by pre-osteoclasts (OCL). This interaction recruits TRAF6 that activates NFκB and c-Fos, the latter dimerizing with c-Jun and forming the AP-1 complex. Both transcription factors cooperate to trigger transcription of NFATc1, which in turn promote its autoamplification. (B) NFATc1, AP-1, PU.1 and MITF cooperate to induce osteclast genes transcription.

clastogenesis, as knock out c-Fos mice develop osteopetrosis due to the lack of osteoclasts (48). AP-1 activation along with a calcium-signal further induces NFATc1 transcription, thus allowing its autoamplification (47).
In cooperation with AP-1, PU.1, NF-κB and MITF, NFATc1 regulates the transcription of several target genes involved in osteoclast differentiation and function. Among them, cathespin K, calcitonin receptor, tartrate-resistant acid phosphatase (TRAcP) (49, 50), β3 integrin and osteoclast-associated receptor (OSCAR) (51) (Figure 3B).

Osteoclast regulation by osteoblasts. The principal regulators of osteoclastogenesis are the osteoblasts. Indeed, the RANKL/RANK signalling relies on the cell-cell interaction between the osteoblasts and the osteoclast precursors. Another key molecule secreted by osteoblasts which interfere with the RANKL pathway is osteoprotegerin (OPG) a decoy receptor for RANKL (52), which has an osteoprotective role. Indeed, OPG is a secreted protein having the same structure of RANK so that it binds to RANKL avoiding its interaction with RANK, with a consequent inhibition of osteoclastogenesis (Figure 3A).
Osteoblasts trigger osteoclastogenesis also by expressing on their membrane surface the M-CSF, which interacts with its receptor c-Fms present on osteoclast precursors thus stimulating their proliferation and osteoclast differentiation (Figure 3A).

Osteoclast regulation by immune system. Several evidence has indicated a tight relationship between the immune system and bone, leading to a new interdisciplinary field, called osteoimmunology, focused to investigate the molecular mechanisms shared by these two tissues (53-55). These findings also point out that the RANKL pathway is necessary but not sufficient to trigger osteoclast differentiation. As described in Figure 4, osteoblasts can regulate osteoclast differentiation by interacting with immunoglobulin (Ig)-like receptors, such as the osteoclast-associated receptor (OSCAR), whose ligand has not yet clearly identified. These receptors are associated with immunoreceptor tyrosine-based activation motif (ITAM)-harbouring adaptor molecules DAP12 and Fc-receptor common γ-sub-

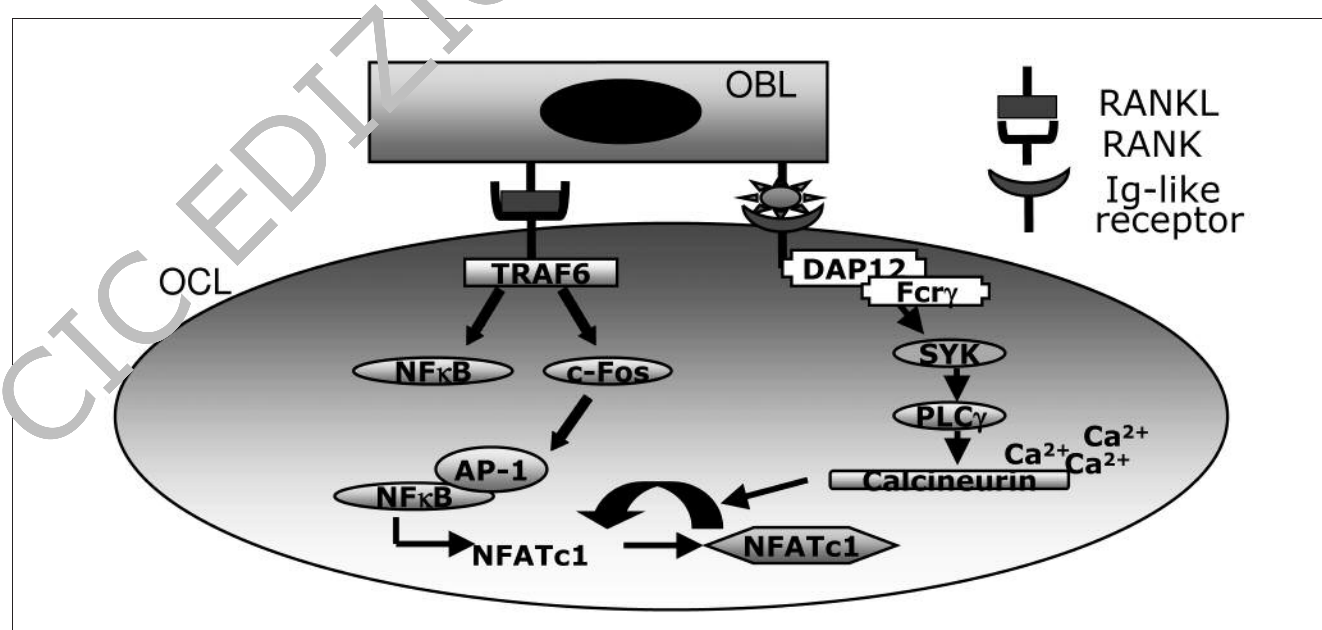

Figure 4 - Schematization of the immunological interplay between osteoclasts and osteoblasts. Osteoblast (OBL) interacts whit Immunoglobulin-like receptor (Ig-like receptor) expressed on pre-osteoclast (OCL) surface, thus allowing the phosphorylation of Dap12 or Fcrg and the subsequent activation of the calcium signalling, which potentiates autoamplification of NFATc1.

unit (FcRg). The role of the latter molecules in osteoclast regulation has been underlined by the evidence that mice deficient in both DAP12 and FcRγ have an osteotropic phenotype (56, 57). Phosphorylation of the ITAM sequence in DAP12 or FcRγ, resulting after RANK activation, allows the recruitment of splenocyte tyrosine kinases (SYK) and the consequent activation of the phospholypase Cγ (PLCγ), which in turn triggers calcium signalling. Calcium signalling promotes osteoclastogenesis by activating the CAMKIV (calcium/calmodulin-dependent protein kinase type IV), which concurs to c-Fos activation, and calcineurin, both cooperating to potentiate NFATc1 auto-amplification (53).

Inflammatory cytokines and osteoclastogenesis. Several findings have demonstrated the involvement of some inflammatory cytokines produced by macrophages, such as interleukin (IL)-1, TNFα and IL-6, in osteoclast differentiation and function (58, 44) thus further supporting the tight relationship between bone and immune system.
TNFα is able to directly stimulate osteoclastogenesis in the presence of M-CSF (59, 60) by stimulating the activation of NF-κB mainly through TRAF2. Moreover, along with TGFβ it induces *in vitro* osteoclastogenesis even in the absence of RANK or TRAF6 (61).
IL-1 is not able alone to induce osteoclast differentiation, but it synergizes with RANKL to induce osteoclastogenesis and bone resorption, probably by stimulating TRAF6. Moreover, it indirectly promotes osteoclastogenesis by stimulating osteoblast secretion of PGE2 and RANKL (44).
Interferon-β (IFN-β) is another cytokine crucial in immune response that negatively affects osteoclatogenesis. Indeed, RANKL signalling induces IFN-β that in turn acts as a negative feedback regulator by inhibiting cFos expression (62).

b) Osteoclast function

Once differentiated, multinucleated osteoclasts need to adhere to the bone matrix and to polarize in order to resorb bone. Indeed, two principal domains can be identified in the osteoclast plasma membrane: the basolateral and the apical domains, which also differ for their function. In the apical domain, it is possible to identify a further membrane specialization, that is the ruffled border, characterized by several folding of the membrane and representing the resorbing organ (51).
One of the earliest events of osteoclast activity is to degrade the inorganic component of the bone matrix, that is the alkaline salts of bone mineral hydroxyapatite. This can be obtained by the release of protons into the area to be resorbed, called resorption lacuna (63, 64). Furthermore, this function also requires sealing the underlined bone matrix, which is obtained through a cytoskeletal rearrangement and the subsequent formation of the actin ring. This is a circumferential structure that surrounds the ruffled membrane and isolates the acidified resorptive microenvironment from the extracellular space. It is formed by several dynamic and dot-like structures called podosomes, each of them consisting of an actin core surrounded by the $\alpha_v\beta_3$ integrin and associated cytoskeletal proteins such as vinculin, α-actinin and talin (65, 66).
As already told, the first step of bone resorption is the release of protons in the compartment between the osteoclast and the bone surface via an electrogenic proton pomp called vacuolar type ATPase (67, 68), which is present both in intracellular vesicles as well as in the ruffled border (67-69). This is a crucial step, as demonstrated by the fact that mutations in the a3 subunit of vacuolar ATPase cause osteopetrosis in humans (70, 71).
The production of protons is ensured by the activity of the carbonic anhydrase II (CA II) (72) which catalyses hydration of CO_2 thus forming carbonic acid (H_2CO_3). H_2CO_3 in turn dissociates to protons (H+) and bicarbonate ions ($HCO3^-$). H^+ are then secreted in the resorption lacuna, while $HCO3^-$ are extruded via an electroneutral chloride/bicarbonate exchanger in the basolateral membrane (73). Moreover, the chloride ion (Cl^-) that enters the cell in exchange of HCO3- are transported into the resorptive microenvironment through a chloride channel (74), thus generating HCl (75). The functional importance of chloride channels was confirmed by the evidence that loss of the chloride channel isoform ClC-7 leads to osteopetrosis in human and mice (76).
Dissolution of mineral crystals allows the digestion of the bone matrix organic component that is performed by matrix metalloproteinases (MMPs) and lysosomial cathepsins. Among the latter, cathepsin K has a crucial role, as its deletion in mice leads to osteopetrosis (77, 78), while mutations in cathepsin K gene lead to pycnodysostosis (79, 80). As far as the MMPs is concerned, osteoclasts mainly produce the MMP-9 isoform and to a lesser extent the MMP-14 (81, 82).

Diseases associated to deregulation of osteoclasts

The correct balance between bone deposition and resorption is crucial for the proper maintenance of the bone mass. Moreover, a complex network of pathways attends the regulation of osteoclasts and osteoblasts activity, as demonstrated in the above paragraphs. Among the bone pathologies, we will briefly describe two opposite diseases, both due to an abnormal osteoclast function. These diseses also mirror the complexity of the mechanisms involved in bone mass regulation.

Rheumatoid arthritis

It is well known that this pathology is due to an inflammation of the synovial membrane with a subsequent destruction of the bone mediated by the osteoclasts (83). However the molecular mechanisms that induce abnormal osteoclast activation have only recently been clarified (53). Cells in the synovium include macrophages, fibroblasts, dendritic cells, plasma cells and most importantly, infiltrated $CD4^+$ T cells, which are a hallmark of the pathogenesis of arthritis (84, 85). Recent reports show that among the latter cells a specific subset, the interleukin-17 (IL17)-producing T helper cells (T_H17 cells) have a crucial role in osteoclast activation (55). These cells do not produce IFNγ, which has an anti osteoclastogenesis activity, while they secrete large amounts of IL-17 that stimulates the expression of RANKL by synovial fibroblasts. IL-17 also acts on macrophages by stimulating their secretion of inflammatory cytokines including TNF, IL-1 and IL-6, which in turn trigger osteoclastogenesis and bone resorption directly or indirectly by stimulating expression of RANKL. Finally, T_H17 cells per se express RANKL (55,86).

Osteopetrosis

Osteopetrosis is a rare genetic disease characterized by an increase of the bone mass due to an inability of osteoclasts to resorb bone (87). According to the way of transmission and to the clinical manifestations, it can be classified into three forms with a wide range of severity: infantile malignant autosomal recessive osteopetrosis (ARO), intermediate autosomal recessive osteopetrosis (IRO), and autosomal dominant osteopetrosis (ADO). In the most severe forms, osteoclast failure does not allows enlargement of bone cavities, thus impairing development of bone marrow, leading to hematological failure. Closure of bone foramina causes cranial nerve compression with visual and hearing deterioration. Patients also present with osteosclerosis, short stature, malformations and brittle bones. This form is fatal in infancy, and is cured with hematopoietic stem cell transplantation, with a rate of success <50% and unsatisfactory rescue of growth and visual deterioration (87).

Loss-of-function mutations of various genes involved in osteoclast function are responsible for this disease. Among them, the TCIRG1 gene, which encode for the a3 subunit of the H+ATPase and accounting for >50% of cases (70, 71), the CLC7 (76, 88) and the OSTM1 genes, which have closely related function and account for approximately 10% of cases (89-91). Further genes are implicated in rare forms with various severities and association with other syndromes and, recently, the RANKL gene was found to be mutated in a subset of patients lacking osteoclasts (92). Autosomal recessive osteopetrosis may also have intermediate severity, with a small number of cases due to loss-of-function mutations of the CAII (93) or the PLEKHM1 genes (94). Dominant negative mutations of the CLC7 gene cause the so-called Albers-Schönberg disease (95), which represents the most frequent and heterogeneous form of osteopetrosis, ranging from asymptomatic to intermediate/severe, thus suggesting additional genetic/environmental determinants affecting penetrance (96).

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