Osteoclast differentiation and activation

William J. Boyle*, **W. Scott Simonet**† & **David L. Lacey**†

**Protein Pathways, Inc., Woodland Hills, California 91367, and* †*Amgen, Inc., Thousand Oaks, California 91320, USA*
(e-mail: bboyle@proteinpathways.com)

Osteoclasts are specialized cells derived from the monocyte/macrophage haematopoietic lineage that develop and adhere to bone matrix, then secrete acid and lytic enzymes that degrade it in a specialized, extracellular compartment. Discovery of the RANK signalling pathway in the osteoclast has provided insight into the mechanisms of osteoclastogenesis and activation of bone resorption, and how hormonal signals impact bone structure and mass. Further study of this pathway is providing the molecular basis for developing therapeutics to treat osteoporosis and other diseases of bone loss.

In life, bone is a rigid yet dynamic organ that is continuously moulded, shaped and repaired. Bone microstructure is patterned to provide maximal strength with minimal mass, as determined by the physiological needs of the organism. How are bone structure and function maintained, and how are changes in bone metabolism induced? Once formed, bone undergoes a process termed remodelling that involves break down (resorption) and build-up (synthesis) of bone; this occurs in micro scale throughout the skeleton. Bone remodelling is the predominant metabolic process regulating bone structure and function during adult life, with the key participant being the osteoclast[1,2]. Imbalances of remodelling can result in gross perturbations in skeletal structure and function, and potentially to morbidity and shortening of lifespan.

Most adult skeletal diseases are due to excess osteoclastic activity, leading to an imbalance in bone remodelling which favours resorption[3]. Such diseases would include osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma and metastatic cancers. For individuals with osteoporosis, bone fractures represent life-threatening events, and today there are in excess of 70 million people worldwide at risk. Recent breakthroughs in our understanding of osteoclast differentiation and activation have come from the analysis of a family of biologically related tumour necrosis factor (TNF) receptor (TNFR)/TNF-like proteins: osteoprotegerin (OPG), receptor activator of nuclear factor (NF)-κB (RANK) and RANK ligand (RANKL), which together regulate osteoclast function[4]. The study of this pathway is providing a deeper understanding of how diverse physiological and pathophysiological signals exert their effects on the RANK signalling pathway to induce osteoclastogenesis, bone resorption and skeletal remodelling, and so control bone mass.

Osteoclastogenesis

The osteoclast is a tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursors cells at or near the bone surface (Fig. 1). A significant breakthrough in our understanding of osteoclastogenesis occurred when murine systems using co-cultures of bone marrow or spleen cells and stromal cells yielded osteoclasts[5]. The close contact between stromal and bone marrow cell types was essential for osteoclastogenesis, and suggested that

stromal-derived factors stimulate this process. It is now known that this system allowed for production of two haematopoietic factors that are both necessary and sufficient for osteoclastogenesis, the TNF-related cytokine RANKL and the polypeptide growth factor CSF-1 (for colony-stimulating factor-1)[6,7], and for the subsequent activation of RANK on the surface of haematopoietic precursor cells[8,9]. Together, CSF-1 and RANKL are required to induce expression of genes that typify the osteoclast lineage, including those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor and the β_3-integrin[7], leading to the development of mature osteoclasts.

The mature, multinucleated osteoclast is activated by signals, which leads to initiation of bone remodelling (Fig. 2). The osteoclast cell body is polarized, and in response to activation of RANK by its ligand[10], it undergoes internal structural changes that prepare it to resorb bone, such as the rearrangements of the actin cytoskeleton and formation of a tight junction between the bone surface and basal membrane to form a sealed compartment. This external 'vacuole' is then acidified by the export of hydrogen ions generated by the ATP6i complex[11]. Secretion continues with the export of the lytic enzymes TRAP and pro-CATK into a resorption pit (Howship's lacunae). Through this process the osteoclast erodes the underlying bone. Degradation products (collagen fragments and solubilized calcium and phosphate) are processed within the osteoclast and released into the circulation. RANKL can activate mature osteoclasts in a dose-dependent manner *in vitro*, and can lead rapidly to the resorption of bone *in vivo* by activating pre-existing osteoclasts[10,12]. The survival of the mature osteoclast, and its participation in successive rounds of bone resorption, is regulated in part by hormones and cytokines[13]. RANKL and interleukin (IL)-1 increase the survival time of the mature osteoclast *in vitro* and *in vivo*, and this may be due to the ability of these factors to induce NF-κB activity[14,15].

At least 24 genes or loci have been shown to positively and negatively regulate osteoclastogenesis and osteoclast activation (Fig. 1), based on naturally occurring mutations or targeted knockout mutations in rodents and humans (see refs 16, 17 for review). Disruption of these genes blocks the development and/or function of the mature osteoclast, resulting in abnormally high levels of mineralized bone and cartilage, a condition called osteopetrosis. The gene products for some of these loci have yet to be identified, although
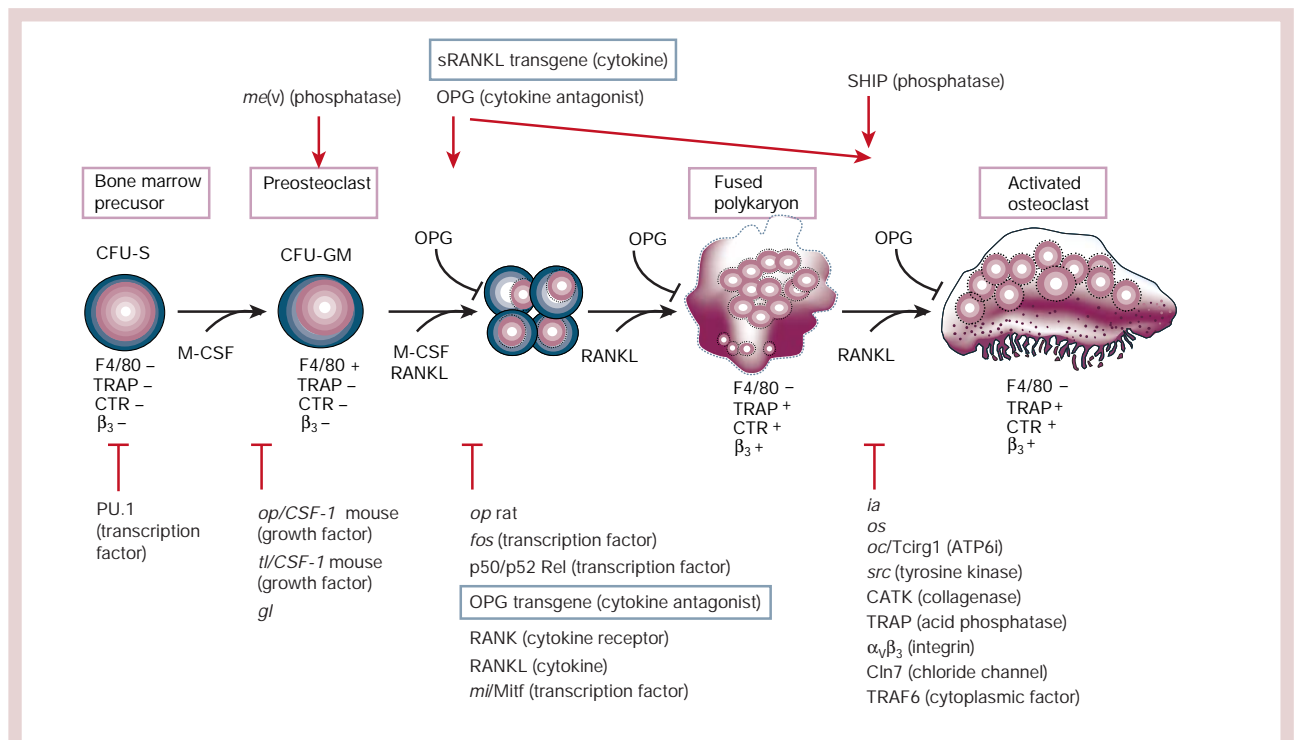
Figure 1 Osteoclastogenesis. Development schema of haematopoietic precursor cell differentiation into mature osteoclasts, which are fused polykaryons arising from multiple (10–20) individual cells. Maturation occurs on bone from peripheral blood-borne mononuclear cells with traits of the macrophage lineage shown below. M-CSF (CSF-1) and RANKL are essential for osteoclastogenesis, and their action during lineage allocation and maturation is shown. OPG can bind and neutralize RANKL, and can negatively regulate both osteoclastogenesis and activation of mature osteoclasts.

Shown below are the single-gene mutations that block osteoclastogenesis and activation. Those indicated in italic font are naturally occurring mutations in rodents and humans, whereas the others are the result of targeted mutagenesis to generate null alleles. Shown above are the single-gene mutant alleles that increase osteoclastogenesis and/or activation and survival and result in osteoporosis. Note that all of these mutants represent null mutations with the exception of the OPG[22] and sRANKL[77] transgenic mouse overexpression models (in blue-outlined boxes).

the functions of the *microphthalmic* (*mi*; transcription factor), *toothless* (*tl*; growth factor) and *osteosclerotic* (*oc*; ATPase) gene products have recently been discovered[17,18]. Targeted disruption of some genes, such as those encoding SHIP (Src homology 2 domain-containing inositol-5-phosphatase) and osteoprotegerin, cause increased osteoclastogenesis and/or activation *in vitro* and *in vivo*, resulting in osteopenia[19,20]. As a class of mutations, these genes exert their effects at various stages of osteoclast development and activation (Fig. 1). Some genes act during the formation and/or survival of the osteoclast precursor cell (*PU.1* and *op/CSF-1*), whereas other genes mediate either the ability of the precursor cell to undergo differentiation (*RANK*, *p50/p52 rel* and *fos*) or the adherence and lytic function of the mature osteoclast (*src*, *oc/Tcirg* and *CATK*). Continued analysis of the unknown loci in this group of genes is likely to further define important control mechanisms within the osteoclast and the osteoclast regulatory networks[21].

The RANKL/RANK/OPG regulatory axis

The bone marrow and stromal cell co-culture system used to generate osteoclasts *in vitro* was also useful for testing the effects of soluble factors on osteoclast formation. A breakthrough in our understanding of how osteoclastogenesis is regulated was the identification of OPG, a soluble protein that blocked osteoclast formation *in vitro* and bone resorption *in vivo*. OPG is a secreted TNFR-related protein that regulates bone density and bone mass in animals[22,23], and upon systemic administration can block pathologic bone resorption in various animal models[22,24]. Because the TNFR motifs of OPG were found to be essential for its biological activity, it was not surprising that the TNF-related surface protein RANKL was identified as a key cytokine that regulated osteoclastogenesis and bone resorp-

tion[6,7,25,26]. RANKL was subsequently shown to bind and activate the TNFR-related protein RANK, a transmembrane signalling receptor[26]. RANK expression on haematopoietic precursor cells is required in the mouse for osteoclast differentiation and activation, and for the resorption of bone and regulation of calcium homeostasis by calcitropic hormones[27,28]. Mice deficient in RANK or RANKL are phenocopies of one another, indicating the essential role of this receptor–ligand pair in bone remodelling[27,29].

The RANKL polypeptide is a type II transmembrane protein found on the surface of expressing cells as a proteolytically released soluble form[7,25,26]. Hormones and factors that stimulate bone resorption *in vivo* induce the expression of RANKL on osteogenic stromal cells[30,31]. RANKL expression by osteoblasts coordinates bone remodelling by stimulating bone resorption by local osteoclasts, which in turn stimulate bone synthesis by closely adjacent osteoblasts by a process called 'coupling'[32]. OPG therefore acts as a decoy receptor by blocking RANKL binding to its cellular receptor RANK. OPG is also produced by osteoblasts in response to anabolic agents such as oestrogens and transforming growth factor-β (TGF-β)-related bone morphogenic proteins (BMPs)[32,33]. OPG overexpression blocks osteoclast production, which leads to osteopetrosis in mice, whereas OPG deletion results in enhanced remodelling of bone and osteoporosis[21,22]. Expression of RANKL and OPG is therefore coordinated to regulate bone resorption and density positively and negatively by controlling the activation state of RANK on osteoclasts.

RANK signalling network in osteoclasts

Activation of RANK by its ligand leads to the expression of osteoclast-specific genes during differentiation, the activation of resorption by mature osteoclasts, and their survival and participation in new
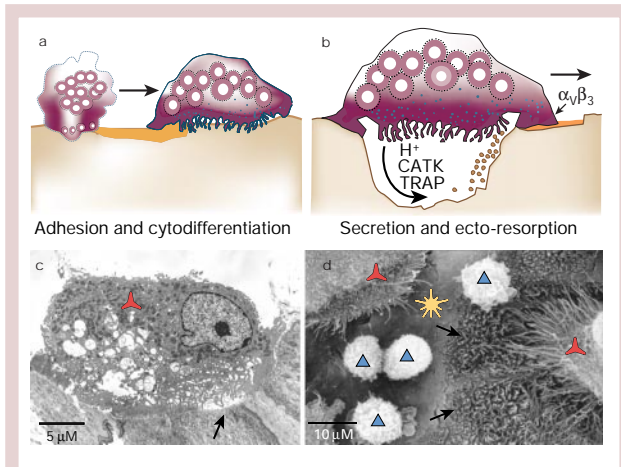
Figure 2 Activation of bone resorption. **a**, Multinucleated polykaryons are recruited by the action of CSF-1 and RANKL, which then adhere to bone and undergo cytodifferentiation into a mature osteoclast. **b**, RANKL stimulates osteoclast activation by inducing secretion of protons and lytic enzymes into a sealed resorption vacuole formed between the basal surface of the osteoclast and the bone surface. Acidification of this compartment by secretion of protons leads to the activation of TRAP and CATK, which are the two main enzymes responsible for the degradation of bone mineral and collagen matrices. **c**, Transmission electron micrograph of an activated mouse osteoclast with a visible ruffled border in a resorption lacunae on the periosteal femoral cortical bone surface. Red propeller, osteoclast; black arrow, a resorption pit. **d**, Scanning electron micrograph of human osteoclasts generated *in vitro* on cortical bone slices from CSF-1- and RANKL-treated peripheral blood mononuclear cells. Red propellers, osteoclasts; black arrows, a resorption pit where the normally smooth lamellar bone surface has been resorbed to expose collagen bundles; yellow star, non-resorbed bone surface; blue triangles, mononuclear cells (potential osteoclast precursors).

rounds of bone degradation at neighbouring sites (Fig. 3). RANK signalling is mediated by cytoplasmic factors that activate downstream signalling pathways that control these various functions. At least five distinct signalling cascades mediated by protein kinases are induced during osteoclastogenesis and activation —inhibitor of NF-κB kinase (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK) and Src pathways (Fig. 3). The key preliminary step in RANK signalling is the binding of TNFR-associated cytoplasmic factors, or TRAFs, to specific domains within the cytoplasmic domain of RANK[9,34,35]. TRAF2, -5 and -6 have all been shown to bind to RANK, and their individual docking sites have been mapped, yet only TRAF6 mutations result in osteopetrosis due to a loss of osteoclast activity[36,37]. RANK mutants that specifically lack TRAF6-binding sites are unable to restore osteoclastogenic potential in RANK^{−/−}-derived haematopoietic precursors[38]. TRAF6 binding to RANK has been modelled, and involves a new structure–function relationship between TRAFs and TNFR-related proteins[39].

TRAF6 acts as a key adaptor to assemble signalling proteins that direct osteoclast-specific gene expression leading to differentiation and activation. The two most closely studied pathways are the activation of transcription factors NF-κB and activator protein-1 (AP-1), whose activities are rapidly induced following ligand binding. Targeted mutagenesis of the p50/p52 component of NF-κB[40,41], as well as the cFos component of AP-1[42], results in osteopetrosis owing to a block in osteoclastogenesis. Activation of these transcription factors can be induced by signalling cascades mediated by protein kinases, including IKK1/2 (NF-κB) and JNK1 (AP-1)[43,44]. Recently, the mitogen-activated protein kinase (MAPK)-related TGF-β-inducible kinase TAK1, along with the TRAF-binding adapter protein TAB2, have been detected in activated receptor complexes[45,46]. Dominant-interfering mutant forms of TAK1 inhibit

RANKL-mediated activation of both IKK1/2 and JNK1, suggesting that TAK1 is important in activation of NF-κB and AP-1[47]. In addition, the MAPK-related kinase MKK7 is required for JNK activation in these cells. It is not yet clear if TAK1 acts directly on IKK1/2 or MKK7, or if other kinases mediate these events (Fig. 3).

The stress-activated protein kinase p38 is also involved in mediating key signals induced by RANK, and is apparently activated via phosphorylation by MKK6[48,49]. Stimulation of p38 results in the downstream activation of the transcriptional regulator mi/Mitf, which controls the expression of the genes encoding TRAP and CATK[50]. mi/Mitf, CATK and TRAP are all required by the mature osteoclast (Fig. 1), indicating the importance of p38 signal transduction. The ERK-1 kinase is also activated by RANK signalling, and seems to be regulated upstream by activation of MEK1[51,52]. The small molecule ERK inhibitors PD98059 and U0126 potentiate RANKL-induced osteoclast differentiation, suggesting that the ERK pathway is involved in negative regulation of osteoclastogenesis.

The Src protein, which is required for osteoclast activation, has also been shown to bind to TRAF6 and to allow RANK-mediated signalling to proceed through the lipid kinase phosphatidylinositol 3-OH kinase (PI(3)K) and the serine/threonine protein kinase AKT[53]. Both PI(3)K and AKT are known to act downstream of Src to induce cell survival, cytoskeletal rearrangements and motility. The lipid kinase SHIP negatively regulates PI(3)K signalling, and SHIP-deficient mice have osteoporosis[19]. The immunosuppressant rapamycin, a small molecule inhibitor of mTOR (FRAP), a downstream substrate of AKT, enhances osteoclastogenesis *in vitro*[54].

Signalling from RANK culminates in the change in gene expression patterns that characterize the active osteoclast. Several groups have used gene expression arrays to map these changes in cell culture systems[55–57]. A consistent finding among these groups is the considerable upregulation of nuclear factor of activated T cells (NFAT)-2, a calcineurin- and calcium-regulated transcription factor. Inhibiting NFAT2 activity using dominant negative alleles blocks osteoclastogenesis, whereas overexpression of the wild-type protein stimulated osteoclast development from embryonic stem cells in a RANKL-independent manner[57]. Similarly, the Myc nucleoprotein factor is induced during differentiation, and its activity is required for *in vitro* osteoclastogenesis[58]. It is not clear which signalling pathway(s) lead to activation of NFAT2 and Myc, although the activation of NFAT2 is blocked by the calcineurin inhibitors cyclosporin A and FK506[57].

Modulation of RANK-induced osteoclastogenesis

There are several levels of control of the RANK signalling pathway that enhance or dampen osteoclastogenesis and activation driven by RANKL. Activation of osteoclast surface receptors for IL-1, c-Fms, TNF-α, PGE2 and TGF-β potentiate osteoclastogenesis *in vitro*, and can stimulate bone resorption *in vivo*. IL1-R and TNFR1 both signal through TRAF6, and activation of these receptors could have a synergistic effect on RANK-mediated TRAF 6 activation[59]. The activation of c-Fms and TGF-β has been reported to upregulate components of the pathway, including the levels of RANK on the cell surface, thereby impacting the potency of RANKL in this system[60–62].

The RANK signalling pathway is negatively controlled by OPG *in vitro* and *in vivo*[22,23]. In addition to this, chemical inhibitors of MEK1 and mTOR lead to increased osteoclastogenesis *in vitro*, suggesting that activation of the Erk and Src pathways can also negatively regulate osteoclastogenesis[52,54]. There is also evidence for feedback mechanisms that switch off the RANK signalling pathway once it is activated. Induction of osteoclastogenesis by RANKL leads to the induction of interferon-β, which is secreted and acts in an autocrine fashion to downregulate the expression of c-Fos, a critical factor involved in osteoclast development[63,64]. Interferon-γ (INF-γ) also has a negative effect on this pathway. Binding of INF-γ to its receptor leads to degradation of TRAF6, and can have an inhibitory effect on osteoclastogenesis *in vitro*[65]. This result is surprising, as INF-γ is an approved therapeutic for the treatment of osteopetrosis, where its
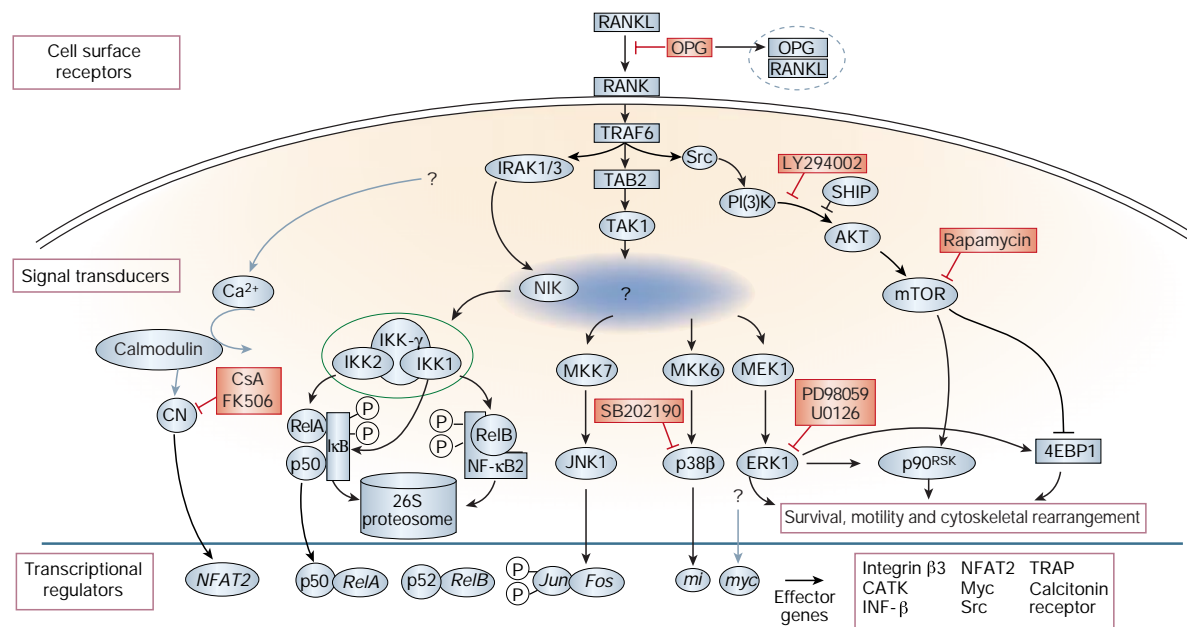
Figure 3 RANK signalling network in osteoclast. Proteins with validated effects involved in RANK signal transduction during osteoclast development and activation, arranged in a signalling cascade from the cytoplasmic membrane to nuclear effectors. RANK and OPG are TNFR receptor-related proteins, and RANKL is a TNF-related cytokine that interacts specifically with either RANK or OPG. Protein nodes are shown in representations of their functional annotation, and are connected by edges (arrows or lines) that denote functional associations depicted in the literature. Red bars indicate target of known small-molecule inhibitors.

effect is to increase bone resorption. The cytokine IL-4 has also recently been shown to negatively regulate osteoclastogenesis, and this process is controlled downstream by STAT (signal transducer and activator of transcription) signalling within the osteoclast[60]. Finally, the binding of calcitonin to its receptor has long been known to dampen osteoclast activation and is the basis for using it as a therapeutic, although its molecular mechanism of action is unclear.

Hormonal control of bone resorption

Certain hormones, cytokines and humoral factors produced in distant organs can also influence bone density and calcium homeostasis locally by inducing RANKL expression within the bone cells (Fig. 4). Most, if not all, calciotropic hormones and pro-resorptive cytokines have been shown to upregulate messenger RNA expression of RANKL in osteoblast cell lines and primary cell cultures[32,33]. OPG, which blocks osteoclastogenesis induced by RANKL, can inhibit osteoclast formation and bone resorption induced by treatment with calciotropic factors, indicating that the RANK signalling pathway in osteoclasts integrates diverse humoral signals that regulate bone resorption and calcium homeostasis. Consistent with this hypothesis, RANK-deficient mice are resistant to bone resorption induced by TNF-α, IL-1β, 1,25(OH)$_2$ vitamin D$_3$ and parathyroid hormone (PTH)-related peptide (PTHrP), which, excluding PTH, are the major calciotropic factors that are known to induce increases in bone resorption and serum hypercalcaemia[27]. The T cell is also an important source of RANKL in the bone[29]. Activation of T cells *in vitro* and *in vivo* leads to increased osteoclastogenesis and bone resorption, suggesting that acute and chronic inflammatory states, and certain leukaemias, contribute to pathologic bone loss[66].

Humoral factors that decrease bone resorption and increase density, such as oestrogens, have a converse effect on the coupling between the osteoblast and osteoclast — OPG expression is increased and/or RANKL expression is decreased, leading to decreased activation of RANK and subsequently the numbers of activated osteoclasts in the bone. Recently the cytokine thrombopoietin, which regulates platelet levels, has also been shown to induce OPG expression in animals,

leading to abnormal increases in bone density[67]. Osteoblast and marrow stromal cells, as well as T cells, therefore act as a key local responding unit within the bone that interpret physiological cues that impact bone remodelling rates, calcium release and bone density (Fig. 4).

Bench to clinic

Except for the recently approved PTH-derived peptide Forteo, current treatments for osteoporosis chiefly retard bone mineral density loss, thereby decreasing the risk for vertebral and long-bone fractures. These compounds target osteoclast-mediated bone resorption and include oestrogens, bisphosphonates and selective oestrogen-receptor modulators (SERMs) (Table 1). Pharmaceutical companies are trying to develop SERMs with improved efficacy and safety profiles, bisphosphonates with more convenient formulations and dosing regimens, and oral forms of calcitonin. Other potential osteoclast targets with chemical inhibitors in preclinical development include the osteoclast-specific protease CATK, the integrin $\alpha_V\beta_3$, and the c-Src tyrosine kinase. All of these osteoclast target molecules are induced during osteoclastogenesis via RANK activation driven by RANKL or RANK-activating antibodies[7,9].

The crucial role of the RANKL/RANK/OPG signalling pathways in regulating bone metabolism is underscored by recent findings that genetic mutations that activate RANK or inhibit the RANKL binding properties of OPG in humans are associated with familial forms of hyperphosphatasia and bone abnormalities[68–72]. Activating mutations in exon 1 of *TNFRSF11A*, the gene encoding RANK, have been found to be associated with osteolytic and non-osteolytic forms of hyperphosphatasia. Expansile skeletal hyperphosphatasia and familial expansile osteolysis are allelic bone diseases caused by overlapping 18-bp and 15-bp tandem duplications in exon 1 of *TNFRSF11A*, respectively, which lengthen the signal peptide of RANK and likely increase its biological activity by sequestering the receptor intracellularly, causing excessive signalling through NF-κB[68,69].

Several mutations in *TNFRSF11B*, the gene encoding OPG, have been found to be associated with idiopathic hyperphosphatasia (also known as juvenile Paget's disease) in humans, an autosomal recessive
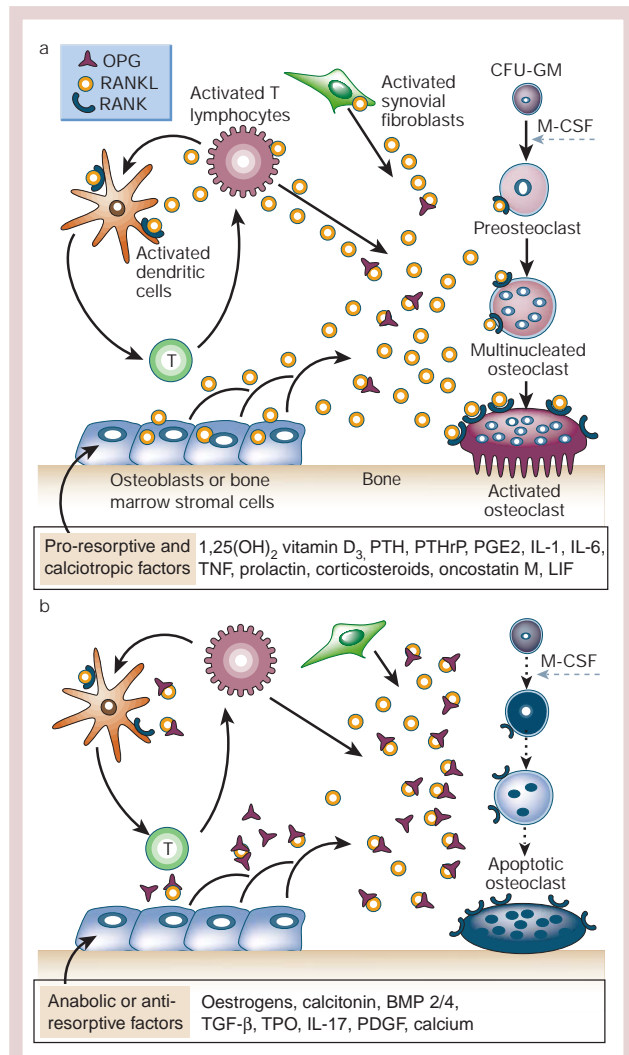
Figure 4 Hormonal control of bone resorption. Schematic representation of the mechanism of action of **a**, pro-resorptive and calcitropic factors; and **b**, anabolic and anti-osteoclastic factors. RANKL expression is induced in osteoblasts, activated T cells, synovial fibroblasts and bone marrow stromal cells, and subsequently binds to its specific membrane-bound receptor RANK, thereby triggering a network of TRAF-mediated kinase cascades that promote osteoclast differentiation, activation and survival. Conversely, OPG expression is induced by factors that block bone catabolism and promote anabolic effects. OPG binds and neutralizes RANKL, leading to a block in osteoclastogenesis and decreased survival of pre-existing osteoclasts.

Table 1 Classes of resorption inhibitors approved for use or in development

Compound class	Type	Mechanism of action
Bisphosphonates	Small molecule	
Amino		Blocks isoprenylation of Rho and Rap and induces apoptosis in osteoclasts
Non-amino		Metabolized to cytotoxic ATP analogues
Oestrogens/SERMs	Small molecule	Oestrogen-receptor agonist
RANKL antagonists	Recombinant protein	Blocks RANKL–RANK interactions
$\alpha_v\beta_3$ antagonists*	Small molecule	Blocks osteoclast adhesion to bone
Src inhibitors*	Small molecule	Blocks steps leading to osteoclast activation
Cathepsin K inhibitors*	Small molecule	Blocks activity of osteoclast-specific collagenase
Calcitonin*	Peptide	Calcitonin-receptor agonist decreases osteoclast activity

An asterix indicates those targets that are induced in osteoclasts by RANKL stimulation, and whose expression and activity is decreased by RANKL blockade. Oestrogen replacement therapy downregulates the level of RANKL expression in bone marrow cells in postmenopausal women[75], and bisphosphonates abrogate the effects of RANK signals that control osteoclast survival and induce cell death[76].

long-acting suppression of bone-turnover markers with a good safety profile[73,74]. Recently, a fully human monoclonal antibody directed against RANKL has entered into early development (P. J. Bekker *et al.*, unpublished data). An appealing aspect of the antibody approach is avoidance of either cross-reacting neutralizing OPG or RANK-activating endogenous antibodies that could lead to safety issues. Whether blocking RANKL leads to clinical benefit will be ultimately addressed in clinical trials.

Future directions

Since the discovery of the RANK/RANKL/OPG signalling axis, our understanding of the osteoclast and its role in bone biology has advanced considerably. Based on the literature, a network of over 60 proteins in the osteoclast are associated with pathways regulating the osteoclast, approximately ten times the number identified three years ago. Using expression data and functional assays, it may be feasible to reconstruct the entire signalling network within the osteoclast, to create mechanistic views of diverse osteoclast functions and their impact on bone remodelling and density. Thus, certain areas of osteoclast biology that remain unclear may be elucidated, leading to the development of therapeutics optimized for both safety and efficacy. □

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bone disease characterized by deformities of long bones and kyphosis[69–71]. Individuals with this disorder exhibit widening of the long-bone diaphyses with a propensity to fracture, accelerated bone turnover, and are typically of short stature. These individuals are phenotypically similar to OPG-knockout mice[20].

The observations that mutations in the genes encoding RANK and OPG cause bone diseases of such striking severity in humans suggest that inhibition of RANKL signalling may be a viable therapeutic strategy for treatment of diseases where excessive resorption or remodelling of bone prevail. In animal models, RANKL antagonists that have shown robust activity include full-length OPG as well as the ligand-binding domains of either OPG or RANK fused to Fc region of immunoglobulin-γ[24]. There is hope that blocking RANKL could preserve bone loss that results from menopause, cancer, inflammation, microgravity/disuse or excesses of either PTH/PTHrP or vitamin D. In the clinic, the Fc-OPG fusion molecules have demonstrated

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