THEMATIC REVIEW

The skeleton: a multi-functional complex organ. The role of key signalling pathways in osteoclast differentiation and in bone resorption

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Abstract

Osteoclasts are the specialised cells that resorb bone matrix and are important both for the growth and shaping of bones throughout development as well as during the process of bone remodelling that occurs throughout life to maintain a healthy skeleton. Osteoclast formation, function and survival are tightly regulated by a network of signalling pathways, many of which have been identified through the study of rare monogenic

diseases, knockout mouse models and animal strains carrying naturally occurring mutations in key molecules. In this review, we describe the processes of osteoclast formation, activation and function and discuss the major transcription factors and signalling pathways (including those that control the cytoskeletal rearrangements) that are important at each stage.

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Introduction

Osteoclasts are required during skeletal development to form and shape bones and throughout life in the process of remodelling that maintains the bone tissue in a healthy state by the continual repair of microfractures (reviewed in Crockett *et al.* (2011b)). Osteoclasts are generally large multinucleated cells, but small, even mononuclear, osteoclasts also exist and can be fully functional. Osteoclast formation and survival and their unique ability to resorb bone are controlled by signalling pathways leading to the activation of transcription factors, with the NFκB pathway being of particular importance. The control of osteoclast differentiation and function by signalling pathways is highlighted by conditions in which key components of such pathways are mutated or absent leading to diseases associated with dramatic changes in osteoclast number or activity (Crockett *et al.* 2011a). In addition, new genetic information links specific signalling pathways to increased risk of developing complex bone diseases such as osteoporosis. In this review, we describe the process of osteoclast formation and resorption, focusing specifically on some of the key signalling pathways involved. This review is not exhaustive; we have not touched on the

important role of osteoclasts in regulating osteoblasts and the signalling pathways involved in this process; we also have not reviewed all the signalling pathways regulating osteoclast function that are shared with the immune system. We encourage the reader to consult other recent reviews in these areas (Edwards & Mundy 2008, Sims & Gooi 2008, Takayanagi 2009).

Osteoclastogenesis

Osteoclasts are formed from myeloid cells of the haematopoietic lineage. In early embryonic life these precursors form in the blood islets in the extra-embryonic tissue. When the embryo becomes vascularised they travel into the embryo proper and establish sites of blood cell formation initially in the foetal liver and spleen and eventually, when endochondral bone formation is complete, they move into the bone marrow space with some blood cell formation also becoming established in sites of intramembranous ossification. Osteoclasts have been successfully cultured from all of these tissues *in vitro* at various stages of embryonic and foetal development in the mouse (Thesingh 1986), as well as from precursors present in the adult mouse circulation (Helfrich *et al.* 1989), and more recently from precursors in the human circulation, which is now common practice in osteoclast research. While we consider osteoclasts derived from all precursors as identical for the purposes of this review, there is increasing information suggesting that perhaps different osteoclast precursors exist in different anatomical sites

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(Everts *et al.* 2009) and this may be related to events very early on in osteoclast ontogeny. Osteoclast development is studied most often in precursor cells derived from bone marrow (mouse) or the circulation (human), while information about mature osteoclasts is often obtained *in vitro* from cells isolated from neonatal rabbits, from *in vivo* model systems and from the study of human bone disease. We review the consensus that has emerged from these studies below.

Osteoclast precursor commitment

Human diseases and genetically engineered or spontaneous mutant rodent lines have demonstrated that deletion of or mutations within any of the genes that encode factors required for commitment to this lineage or for the activation of mature osteoclasts result in the high bone mass condition osteopetrosis (Villa *et al.* 2009). Commitment of cells to the myeloid lineage (see Fig. 1A) is determined through the expression of transcription factor PU.1, a member of the ETS family of transcription factors (Scott *et al.* 1994). Deletion of PU.1 in mice results in a complete lack of differentiation of cells to the monocyte/macrophage lineage and causes osteopetrosis as a result of lack of osteoclast formation (Tondravi *et al.* 1997). Many genes required for osteoclast formation and function have PU.1 binding sites within their promoters (Luchin *et al.* 2000, 2001, Matsumoto *et al.* 2004, Kim *et al.* 2005). Importantly, in the early stages of haematopoietic stem cell commitment to the monocyte/macrophage lineage, PU.1 stimulates the expression of CSF1R, the receptor for CSF1, commonly referred to as macrophage colony-stimulating factor (M-CSF; DeKoter *et al.* 1998). M-CSF and CSF1R are well established as critical components required for the generation of osteoclasts. Animal models in which CSF1R is knocked down develop severe osteopetrosis as a consequence of reduced osteoclast numbers. A similar osteopetrotic phenotype is observed within naturally occurring mutations in M-CSF as in the *op/op* mouse or the *tl/tl* rat, highlighting the critical role of this pathway in commitment to the monocyte lineage (Felix *et al.* 1990b, Yoshida *et al.* 1990, Wojtowicz *et al.* 1997, Dai *et al.* 2002).

The osteoclast differentiation defect in *op/op* mouse can be fully rescued by injecting M-CSF (Felix *et al.* 1990a), and the monocyte defect can be overcome by expressing the pro-survival factor BCL-2 (Lagasse & Weissman 1997). Signalling through CSF1R induces expression of receptor activator of NFκB (RANK; TNFRSF11A) via up-regulation of the transcription factor c-FOS. In addition, RANK expression is controlled by PU.1 in cooperation with other transcription factors (Kwon *et al.* 2005). Elegant animal studies have revealed the critical role for activator protein 1 (AP-1) in osteoclastogenesis. The AP-1 heterodimeric transcription factor complexes comprise members of the Fra, Fos, Jun and activating transcription factor (ATF) families. Deletion of the *c-Fos* gene results in osteopetrosis by halting osteoclast differentiation at the macrophage stage and this defect can be completely rescued by expressing FOS protein (Grigoriadis *et al.* 1994). Despite the

Figure 1 The transcription factor PU.1 is responsible for committing haematopoietic stem cells to the monocyte/macrophage lineage (osteoclast precursors). (A) PU.1 stimulates the expression of the CSF1R receptor in the osteoclast precursors. Macrophage colony-stimulating factor (M-CSF) signalling is activated by M-CSF to promote macrophage proliferation. Macrophage colony-stimulating factor (M-CSF) signalling is activated by M-CSF to promote macrophage proliferation. Microphthemia-induced transcription factor (MITF) is up-regulated by M-CSF and through the induction of BCL-2 stimulates macrophage survival. M-CSF and PU.1 also up-regulate expression of receptor activator of NFκB (RANK). (B) Activation of the RANK receptor through binding of RANK ligand (RANKL) results in the recruitment of TRAF6 to the cytoplasmic domain of RANK. A K63-polyubiquitin chain (green) forms on TRAF6, which is stabilised by the scaffold protein p62, to create a platform for the assembly of the TAK1/TAB2 complex. Phosphorylation of TAK 1 results in the K63-polyubiquitination of NEMO and the subsequent phosphorylation of IKKα that phosphorylates the inhibitory protein IκBα, targeting it for K48-ubiquitination (red) and degradation. In addition, RANKL activates MAP kinase pathways resulting in the phosphorylation of ERK1/2 and c-jun N-terminal kinase (JNK) that activate AP-1 transcription factors and p38 that activates MITF. Following activation, the NFκB, AP-1 and MITF transcription factors enter the nucleus to regulate the expression of genes required for osteoclast differentiation.

critical role for c-FOS in osteoclastogenesis, its precise binding partners *in vivo* have yet to be determined but it is most likely to interact with Jun family members. FRA-1 also plays a role in osteoclast differentiation (Wagner & Eferl 2005) where FRA-1 can substitute for c-FOS deficiency but not when partnered with Jun proteins and indeed, as with c-FOS, the exact binding partner for FRA-1 remains to be identified (Bakiri *et al.* 2007).

Microphthalmia-induced transcription factor (MITF) is another key transcription factor important in the late stages of osteoclastogenesis that becomes activated in response to the initiation of signalling via the CSF1R and RANK receptors. Naturally occurring mutations in *Mitf* (*mi/mi* mouse) cause severe osteopetrosis as a result of reduced osteoclast formation and fusion (Murphy 1973, Thesingh & Scherft 1985) whereas varying degrees of osteopetrosis result from other mutations within the protein (Sharma *et al.* 2009). Activation of MITF in response to M-CSF induces BCL-2 expression and promotes macrophage survival (McGill *et al.* 2002). In addition, MITF in conjunction with PU.1 up-regulates the expression of the critical osteoclast receptor RANK via MITF binding sites within the RANK promoter (Ishii *et al.* 2008). There are three isoforms of MITF but only one, MITF-E, is up-regulated during osteoclastogenesis (Lu *et al.* 2010). In macrophage precursors, association of MITF and PU.1 with corepressors, including EOS, an Ikaros family member, prevents the activation of osteoclast-specific genes. During osteoclast differentiation, however, EOS expression decreases allowing MITF and PU.1 to become active and up-regulate osteoclastogenesis (Hu *et al.* 2007). MITF and PU.1 regulate other transcription factors that control osteoclast formation and activation through binding to various promoter sites. These include the up-regulation of osteoclast critical genes including nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), vacuolar ATPase and cathepsin K (Sharma *et al.* 2007). Indeed, promoter reporter assays demonstrated that cathepsin K promoter activation is prevented in the presence of MITF containing osteopetrosis-causing mutations and cathepsin K expression is lower in cells isolated from *mi/mi* mice (Motyckova *et al.* 2001).

Formation of mature osteoclasts

RANK ligand (RANKL; TNFSF11) is expressed by osteoblasts and stromal cells. Activation of RANK signalling by RANKL (via the pathway illustrated in Fig. 1B) leads to expression of genes required for the fusion of mononuclear osteoclast precursors, such as dendritic cell specific transmembrane protein (*DC-STAMP*), as well as of genes required for regulating the resorptive capacity of multinucleated osteoclasts, including those encoding vacuolar ATPase, CLC-7, cathepsin K, MMP9 and calcitonin receptor (Crockett *et al.* 2011b). During this maturation process, the cells become quiescent, lose the ability to proliferate and are committed to become osteoclasts (Fig. 1B). Recent evidence has emerged for the presence of circulating quiescent osteoclast precursors that already express RANK, suggesting that the early stages of commitment to the lineage take place

at sites distant from bone, for example in the spleen, and that such committed cells can immediately differentiate into osteoclasts upon exposure to RANKL when they enter the bone marrow (Takahashi *et al.* 2010). It has been suggested that entry of the committed osteoclast precursors into the bone remodelling sites is controlled by quiescent osteoblasts (perhaps bone lining cells) that form a canopy over the bone remodelling compartment. Such canopies are disrupted in diseases associated with dysfunctional bone turnover such as osteoporosis and myeloma (Andersen *et al.* 2010).

Deletion of RANK (*Tnfrsf11a*) or RANKL (*Tnfrsf11*) in mice and presence of loss-of-function mutations in humans have been associated with osteoclast-poor osteopetrosis caused by lack of osteoclast formation in both mice and humans (Dougall *et al.* 1999, Hsu *et al.* 1999, Kong *et al.* 1999, Sobacchi *et al.* 2007, Guerrini *et al.* 2008). In addition, deletion of RANK signalling intermediates (Fig. 1B), including TNFR-associated factor 6 (TRAF6) and NFκB induce an osteopetrotic phenotype (Iotsova *et al.* 1997, Lomaga *et al.* 1999). RANK is a type I transmembrane receptor belonging to the TNF receptor superfamily. The crystal structure of the extracellular domain of RANK has recently been described in the presence and absence of RANKL (Ta *et al.* 2010). These studies showed tight binding between RANK and RANKL and illustrate how single base changes to the receptor-binding domain in RANKL drastically reduce interaction with RANKL and prevent osteoclast formation. Like other members of the TNF family, RANK is a trimeric protein. Binding of the trimeric ligand RANKL, results in activation of the transcription factor NFκB (Figs 1B and 2). NFκB proteins are hetero- or homodimeric transcription factors, consisting of p65, p50, p52 and RELB subunits. The critical importance of this transcription factor for osteoclasts is highlighted by the phenotype of mice that lack the p50 or p52 subunit and are unable to form osteoclasts (Franzoso *et al.* 1997, Iotsova *et al.* 1997). RANK itself does not possess any kinase activity and relies on signal transduction via the recruitment of other signalling factors to the C-terminal domain. TRAF6 binds to distinct regions of the cytoplasmic domain of RANK (Fig. 1B). Specific deletion of these regions results in a failure to activate NFκB and inhibits osteoclastogenesis *in vitro* (Darnay *et al.* 1999, Armstrong *et al.* 2002). Although there are consensus binding sites for other TRAFs in RANK, these sites are not essential for NFκB activation in osteoclasts as deletion of *Traf2* and *Traf5* in mice does not lead to osteopetrosis (Galibert *et al.* 1998). The signalling events that follow the recruitment of TRAF6 to RANK are complex: a scaffold of proteins forms with TRAF6 that recruits atypical protein kinase C (αPKC) and facilitates the ubiquitination of TGFβ activating kinase (TAK1) and the two adaptor molecules TAB1/2, which in turn activate the inhibitory kappa B kinase (IKK) complex resulting in the phosphorylation of the inhibitory kappa B alpha (IκBα) which releases NFκB to translocate to the nucleus (Fig. 2) and activate transcription of pro-osteoclastogenic

Figure 2 NFκB translocates from the cytoplasm to the nucleus of osteoclasts when stimulated with RANKL. Human osteoclasts were serum starved for 3 h then stimulated with 50 ng/ml RANKL for 0, 15 and 30 min. The cells were then fixed in 4% paraformaldehyde and immunostained for the NFκB family member p65 (A–C) and the nuclei counterstained with DAPI (D–F). Cells showing nuclear p65 are highlighted (*) in panels A–C.

genes (Wang *et al.* 2001) as shown in Fig. 3. In addition to NFκB, RANK has been shown to activate MAP kinase signalling pathways through ERK1/2 and c-jun N-terminal kinase (JNK), which activate AP-1 transcription factors, and p38 which activates MITF (Lee & Kim 2003) and thus regulates osteoclast differentiation. The C-terminal tail of RANK recruits c-SRC which activates the family of Cbl proteins known to regulate osteoclast survival perhaps via regulation of RANK receptor recycling and degradation (Arron *et al.* 2001, Horne *et al.* 2005). These signalling molecules are also important for the activation of resorption, as discussed below. The target gene for NFκB and c-FOS in osteoclast precursors is NFATc1 (Fig. 3), the master transcription factor in osteoclastogenesis (Takayanagi *et al.* 2002). NFATc1 is induced early in osteoclast differentiation in response to increased levels of intracellular calcium that activate calcineurin. The exact mechanisms that lead to the rise in calcium levels are not known but may involve activation of PLCγ and production of diacylglycerol and IP3 that releases calcium from the endoplasmic reticulum. Calcineurin is activated to dephosphorylate cytosolic NFATc1, which then translocates to the nucleus and regulates the expression of osteoclast-specific genes. NFATc1 binds in concert with PU.1 and MITF to the promoter regions of essential genes for osteoclast function encoding for proteins such as cathepsin K, OSCAR, DC-STAMP, TRAP and V-ATPase-d2. To further enhance the level of NFATc1

expression required to regulate osteoclast formation, NFATc1 also up-regulates its own expression in a process of autoamplification (Asagiri *et al.* 2005).

There are numerous factors that modulate RANKL-stimulated osteoclast formation. Potentiators include immune factors such as tumour necrosis factor α (TNFα) and interleukin 1 (IL1). These factors are up-regulated in bone-destroying diseases such as rheumatoid arthritis associated with localised bone erosions (Adamopoulos *et al.* 2006). Inhibitors include osteoprotegerin (OPG; *TNFRSF11B*), a decoy receptor for RANKL and negative regulator of osteoclast formation *in vivo* (Simonet *et al.* 1997). Inactivating mutations in this gene cause Juvenile Paget's disease, a syndrome that is characterised by increased bone turnover featuring focal areas of increased bone remodelling (Whyte *et al.* 2002). In addition to the central and indispensable RANK signalling networks that regulate osteoclast formation, the whole process is also regulated by the activities of other important factors. For example, the ratio of two

Figure 3 Upon activation of CSF1R and RANK, a number of pro-osteoclastic transcription factors are activated to up-regulate the expression of several genes critical for osteoclast differentiation and survival. Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) is one of the main targets of RANK signalling and is strongly up-regulated following the binding of NFκB and NFATc2 to the NFATc1 promoter. NFATc1 expression is further enhanced by several other transcription factors including the members of the AP-1 complex and CREB-binding protein 1 (CBP) which is recruited via elevated cytosolic calcium levels. NFATc1 also undergoes autoamplification binding to its own promoter following the initial NFκB-induced expression. NFATc1 in conjunction with members of the AP-1 complex, microphthalmia-induced transcription factor (MITF) and PU.1 bind to promoter regions of several genes that encode proteins involved in osteoclast differentiation, function and survival including tartrate resistant acid phosphatase (TRAP), osteoclast-associated receptor (OSCAR), calcitonin receptor, cathepsin K, CLC-7 and β₃ integrins. Expression of these genes is tightly regulated and is down-regulated by MAFB. This factor inhibits the pro-osteoclastic transcription factors preventing them from binding to the promoter regions of target genes. The activity of MAFB is regulated by the ratio of LIP to LAP and when this ratio is altered in favour of LIP the activity of MAFB is inhibited and osteoclast gene expression is up-regulated.

isoforms of the transcription coactivator C/EBP β control osteoclast formation. The dominant short C/EBP β isoform (LIP) has a shortened N-terminus as a result of translation initiation from an alternative site. Translation from the alternative start site is dependent on the activation of mechanistic target of rapamycin (MTOR; also known as mammalian target of rapamycin, mTOR). The longer LAP isoform contains an N-terminal transactivation domain that increases expression of MAFB that decreases osteoclast differentiation by inhibiting several pro-osteoclastic transcription factors including MITE, PU.1, NFATc1 and AP-1 (Kim *et al.* 2007). By contrast, LIP inhibits MAFB expression, allowing osteoclast formation to proceed. Therefore, through modulation of the MTOR signalling pathway, the ratio of LIP to LAP is altered (Fig. 3) and the level of osteoclastogenesis controlled (Smink *et al.* 2009).

Other regulators can be found in the CCN proteins, a family of secreted matrix proteins that act as multifunctional signalling regulators (Perbal 2004). CCN1 inhibits osteoclast formation *in vitro* (Crockett *et al.* 2007) via a mechanism that may involve a paracrine effect of osteoblast-derived CCN1 (Wang & Crockett, unpublished observations) whereas CCN2 has been shown to be elevated in the serum and synovium of patients with rheumatoid arthritis where it is expressed in the synovial macrophages and is associated with increased osteoclast formation and activity (Nozawa *et al.* 2009), possibly as a consequence of regulating DC-STAMP expression (Nishida *et al.* 2011).

Reduced oxygen tension stimulates osteoclast formation *in vitro* with little or no direct effect on osteoclast activation. This may be important in the pathology of conditions that affect the vasculature resulting in reduced tissue perfusion, many of which are also associated with increased bone loss (Arnett *et al.* 2003). By contrast, acidification of cultures of mature osteoclasts *in vitro* leads to increased osteoclast activation, whilst reduced pH during the formation of osteoclasts does not affect osteoclast number (reviewed in Arnett (2010)). Extracellular nucleotides are also likely local regulators of osteoclast formation and/or activity since multiple P2 receptors including, P2Y1, P2Y6 and P2X7, have been shown to play roles at different stages of the process *in vitro* (Hoebertz *et al.* 2001, Grol *et al.* 2009, Orriss *et al.* 2011b) and manipulation of receptor expression *in vivo* results in distinct bone phenotypes (Orriss *et al.* 2011a). However, the relative contribution of each receptor subtype to the overall maintenance of osteoclasts *in vivo* and indeed the coordinated process of bone remodelling is not yet known and there is likely to be redundancy between them (Orriss *et al.* 2010).

Osteoclastic bone resorption

Once formed, osteoclasts have the unique ability to destroy (resorb) mineralised bone as part of bone modelling in early life, or as part of remodelling during adult life (Crockett *et al.* 2011b). This process occurs in three stages: osteoclast

attachment and polarisation, initiation of resorption and cessation of resorption (perhaps followed by cell death). The tight regulation of these stages is critical, since too much bone resorption leads to a reduction in the overall amount of bone such as found, for example, in postmenopausal osteoporosis. Too little resorption, on the other hand, causes osteopetrotic conditions where bone mass is high. Both high and low bone mass conditions can result in increased susceptibility to fractures, in the latter because there simply is too little bone and in the former because the bone quality and strength is poor. This high risk of fracture is perhaps the defining characteristic of bone diseases caused by deregulation of osteoclast activity since high bone mass conditions caused by increased osteoblast activity are not associated with increased risk of fracture.

Initiating resorption

When osteoclasts attach to bone, they develop integrin-mediated adhesion structures called podosomes that are reorganised into a densely packed ring, the sealing zone (Jurdic *et al.* 2006, Luxenburg *et al.* 2007). This area of tight attachment to the extracellular matrix effectively seals off a compartment beneath the cell where bone degradation occurs (Luxenburg *et al.* 2007). Indeed as a result of polarised vesicular trafficking, the membrane enclosed by the sealing zone becomes a highly convoluted ruffled border, where protons and proteases are secreted to demineralise and degrade the bone matrix respectively (Fig. 4A; Lakkakorpi & Väänänen 1996, Palokangas *et al.* 1997, Mulari *et al.* 2003a). Therefore, osteoclast adhesion mediated by the engagement of integrins and CD44 receptors induces signalling pathways necessary to initiate resorption.

Podosomes are formed by a core of densely packed actin filaments and F-actin-associated proteins such as cortactin, Wiskott-Aldrich syndrome proteins (WASp) and ARP2/3, surrounded by integrins and attachment-related proteins such as vinculin and talin as illustrated in Fig. 5 (Jurdic *et al.* 2006, Spinardi & Marchisio 2006). These structures are highly dynamic and can assemble and disassemble within minutes in contrast to more stable structures of focal adhesions such as those seen in other cell types (Destaing *et al.* 2003, Block *et al.* 2008). Osteoclast attachment to the bone surface is mainly dependent on integrins, particularly the vitronectin receptor ($\alpha_v\beta_3$), the most abundant integrin in osteoclasts (Davies *et al.* 1989, Nakamura *et al.* 1999, McHugh *et al.* 2000). The deletion of the β_3 subunit in mice has dramatic effects on osteoclast spreading, cytoskeletal organisation and activity, and, as expected, leads to a progressive osteopetrosis (McHugh *et al.* 2000). Upon binding to RGD motif-containing ligands, such as vitronectin (Humphries *et al.* 2006), $\alpha_v\beta_3$ integrins recruit numerous proteins, including the non-receptor tyrosine kinases PYK2, SRC and SYK, as well as the ubiquitin ligase c-CBL (Duong *et al.* 1998, Sanjay *et al.* 2001, Zou *et al.* 2007). All members of this complex are involved in both podosome assembly and disassembly

Figure 4 (Left panel) Transmission electron micrograph of an osteoclast illustrating its attachment to the bone surface. The clear zone (CZ) rich in F-actin is indicated as is the ruffled border membrane (RB). Bar is 5 μ m. The many proteins present in these membrane domains are detailed in Fig. 5. (Right panels) Confocal micrographs of immunofluorescently stained osteoclasts cultured on dentine, illustrating the formation of podosomes and F-actin rings. Osteoclasts were treated with calcitonin to disrupt attachment to bone and then allowed to repolarise. In i) cells have started to reform podosomes (arrow) and these are beginning to aggregate (2 h after removal of calcitonin) and ii) the osteoclast has reformed F-actin rings (single arrows), still reforming a third F-actin ring (double arrow) and is actively resorbing again (6 h after removal of calcitonin). Bar is 20 μ m. Osteoclasts were seeded on slices of dentin (black) and stained for F-actin (red), plasma membrane (blue) and nuclei (green).

as well as podosome organisation (Horne *et al.* 2005, Zou & Teitelbaum 2010).

The critical role of SRC in the assembly, organisation and stability of podosomes is underlined by the findings that osteoclasts derived from SRC-deficient mice, that have an osteopetrotic phenotype, have fewer podosomes (Destaing *et al.* 2008) and that SRC kinase activity is required for actin ring formation and resorption (Miyazaki *et al.* 2004). SRC kinases phosphorylate numerous substrates including cortactin and gelsolin that regulate actin polymerisation and podosome turnover (De Corte *et al.* 1997, Tehrani *et al.* 2007). Cortactin depletion leads to loss of podosomes that can be rescued by re-expressing wild-type cortactin in the cells, however no rescue was observed when a mutant of cortactin that cannot be phosphorylated by SRC was used (Tehrani *et al.* 2006). This demonstrates the importance of SRC phosphorylation for podosome formation. Similar to cortactin, gelsolin deficiency also inhibits podosome formation and osteoclast migration (Chellaiah *et al.* 2000). SRC also phosphorylates spleen tyrosine kinase (SYK), which leads to cytoskeletal reorganisation by promoting the activation of VAV3, a Rho family guanine exchange factor (Faccio *et al.* 2005), and subsequently of RAC and CDC42 (Itzstein *et al.* 2011, Teitelbaum & Zou 2011). These small GTPases stimulate WASp and cortactin, known activators of the ARP2/3 complex that is required for actin nucleation and polymerisation (Higgs & Pollard 2001, Weaver *et al.* 2001, Itzstein *et al.* 2011). M-CSF also plays a role in integrin

signalling by promoting a change in conformation of β_3 integrin, increasing its affinity for ligand (Faccio *et al.* 2002, Teitelbaum & Zou 2011) and by stimulating Rho family-mediated cytoskeletal rearrangement (Faccio *et al.* 2005, Itzstein *et al.* 2011).

Recently, it was discovered that mutations in kindlin-3, a protein that activates integrins on blood-derived lymphocytes and platelets (Moser *et al.* 2008), result in osteopetrosis (Schmidt *et al.* 2011). Osteoclasts generated from mice deficient in kindlin-3 lack podosomes and are unable to activate signalling downstream of β_1 , β_2 and β_3 integrins (Schmidt *et al.* 2011), confirming the major role of integrin signalling for osteoclast adhesion and polarisation.

Osteoclast attachment to bone matrix also involves the hyaluronic acid receptor CD44 that binds to osteopontin (Goodison *et al.* 1999). Similarly to integrin signalling, the stimulation of CD44 activates ARP2/3 complex and WASp-interacting protein (WIP) deficiency in osteoclasts leads to a loss of podosomes that can be rescued by osteopontin treatment (Chabadel *et al.* 2007), demonstrating that CD44-mediated signalling pathways are required for podosome formation and osteoclast attachment to bone matrix.

Podosome rearrangement into a densely packed ring is critical for the formation of a sealing zone (Figs 4B and 5). Unlike osteoclasts seeded on plastic or glass, only osteoclasts plated on bone matrix form a sealing zone. Saltel *et al.* (2004) demonstrated that adhesion of osteoclasts to apatite matrix activates SRC and Rho signalling triggering the formation of the sealing zone. Indeed, this process is characterised by the stabilisation of the microtubule network (Destaing *et al.* 2005, Jurdic *et al.* 2006). In osteoclasts, deacetylation of microtubules by the histone deacetylase HDAC6 is promoted by increased RHO activity (Destaing *et al.* 2005) and leads to the disruption of the sealing zone. PYK2 down-regulates RHO activity to allow the stabilisation of the microtubules and the sealing zone (Gil-Henn *et al.* 2007). Recently, it has been demonstrated that CBL proteins protect microtubules from deacetylation by competing with HDAC6 for binding to β -tubulin (Purev *et al.* 2009).

The ruffled border (Figs 4A and 5) is formed by polarised transport of acidic vesicles of the endocytic/lysosomal pathway to allow the delivery of the resorption machinery such as cathepsin K, vacuolar ATPase and the chloride/ H^+ antiporter CLC-7 to the plasma membrane in direct contact with bone (Coxon & Taylor 2008). This vesicular trafficking is regulated by RAB small GTPases including RAB7 and RAB3D since ruffled borders are absent in osteoclasts lacking either protein (Zhao *et al.* 2001, Pavlos *et al.* 2005, Itzstein *et al.* 2011). It has been hypothesised that the interaction of RAB7 with RAC1 is required for the transfer of vesicles from microtubules to cortical actin and their subsequent delivery and fusion to the plasma membrane (Sun *et al.* 2005, Frasa *et al.* 2010). The V-ATPase is also a potential mediator of this process via its direct binding to actin (Zuo *et al.* 2006) since mutations in the *TCIRG1* gene which encodes the α_3 subunit of V-ATPase cause osteopetrosis due not only to defects in

Figure 5 Podosomal signalling and vesicular trafficking pathways are necessary for osteoclast polarisation and resorption. Following contact with bone, the osteoclast becomes polarised and forms several distinct regions including the sealing zone (SZ), the ruffled border (RB) and the functional secretory domain (FSD). The ruffled border is a highly convoluted membrane that forms an isolated area where resorption of the bone matrix takes place. During polarisation, the osteoclast rearranges its actin cytoskeleton to form an F-actin ring (SZ) comprised of highly dynamic podosomes that facilitate its attachment and detachment to and from the bone surface. The formation of podosomes is induced by $\alpha_v\beta_3$ integrin and CD44 signalling to activate SRC kinase and ARP2/3 complex, respectively, thereby regulating actin polymerisation. The regulation of RHO activity by PYK2 as well as the competition of CBL with HDAC6 to bind to β -tubulin allows the stabilisation of the microtubule network required for the SZ. The ruffled border provides a large surface area for the release of proteolytic enzymes (cathepsin K) and protons required for the degradation of the bone matrix. During resorption, acidic vesicles are targeted to the RB by RAB small GTPases and are inserted into the RB membrane where the vacuolar-type proton ATPase (V-ATPase) and chloride/H⁺ antiporter (CLC-7) transport protons and chloride ions whilst maintaining electroneutrality. The products of resorption are removed from the resorption area via transcytosis through the osteoclast where they are secreted across the FSD.

acidification of the resorption lacuna but also in ruffled border formation (Blair *et al.* 2004, Ochotny *et al.* 2011). The role for RAB7 in ruffled border formation is also supported by the observation that RAB7 interacts with PLEKHM1, a large cytosolic protein in which mutations cause a loss of ruffled border resulting in osteopetrosis caused by dysfunctional osteoclasts (Van Wesenbeeck *et al.* 2007). The fusion of the vesicles to the plasma membrane could be mediated by synaptotagmin (SYT) proteins since osteoclasts lacking SYT VII have defects in ruffled border formation and cathepsin K secretion suggesting that this family of proteins regulate protease exocytosis at the ruffled border (Zhao *et al.* 2008).

Once degraded, bone matrix products are internalised by endocytosis in the central area of the ruffled border where vesicle coat proteins such as clathrin and adaptor protein complex 2 (AP-2) are localised as well as the GTPase dynamin that mediates pinching off of vesicles (Mulari *et al.* 2003b).

The vesicles are then targeted to the functional secretory domain (Fig. 5) in the centre of the basolateral domain to be secreted (Mulari *et al.* 2003a,b), however the regulation of this transcytosis process remains to be determined.

Switching off resorption

Osteoclasts are highly motile cells and migrate from one site of resorption to a new site. The capacity of the cells to fulfil this role is made possible by the ability to disassemble and reassemble their podosomes quickly. Indeed, high levels of phosphorylated cortactin in osteoclasts are associated with low levels of actin turnover whereas unphosphorylated cortactin is associated with increased podosome dynamics (Luxenburg *et al.* 2006). Cortactin may form a link between the podosomes and the microtubule network since a recent report suggests that it interacts with EB-1, a protein that is

associated with the '+' ends of microtubules and regulates stabilisation of microtubules (Biosse-Duplan *et al.* 2011).

The exact signals that halt resorption by an individual osteoclast in order for it to move to another location are not known. As described above, control of resorption at all stages is required to maintain bone quantity and quality and there are a number of physiological and pharmacological regulators of this process. Calcitonin is a hormone that is released by the thyroid parafollicular C-cells in response to raised calcium levels in the circulation (normally maintained to within 2.2–2.6 mM). Although mature osteoclasts express calcitonin receptors and respond rapidly to calcitonin which reversibly inhibits osteoclast activity via activation of cAMP (Chambers & Moore 1983, Hattersley & Chambers 1991, reviewed in Inzerillo *et al.* (2002)) the exact physiological role for calcitonin in controlling resorption in humans is not known. Calcitonin is, however, a very useful tool with which to study the ultrastructural changes that occur during osteoclast activation (Fig. 4B). In postmenopausal osteoporosis, the overall balance of formation to resorption tips in favour of resorption resulting in low bone mineral density and increased occurrence of pathological fractures. This can be linked to the loss of oestrogen at this time since this sex hormone is likely to play a role in osteoclast survival and induces osteoclast apoptosis *in vivo* and *in vitro* (Hughes *et al.* 1996). The exact mechanisms by which oestrogen mediates these effects is not known but may be via a paracrine effect of oestrogen acting via oestrogen receptor-mediated expression of FAS-ligand on osteoblasts (Krum & Brown 2008). Bone loss following the menopause can be prevented by treatment with potent nitrogen-containing bisphosphonates, a class of drugs that target to bone and inhibit bone resorption via inhibition of the mevalonate pathway (Rogers *et al.* 2010). This inhibition prevents the proper processing (prenylation) of small GTPases that, as described above, are absolutely critical for resorption (Itzstein *et al.* 2011). Strontium ranelate also prevents bone loss but in addition stimulates new bone formation and reduces the risk of osteoporotic fracture (Kanis *et al.* 2011). The mechanisms by which this divalent cation achieves this are not fully understood but may involve both calcium-sensing receptor dependent and independent processes that lead to osteoclast apoptosis and stimulate osteoblast differentiation (Fromiguet *et al.* 2009, Hurtel-Lemaire *et al.* 2009, Caudrillier *et al.* 2010).

Although overall osteoclast survival is critically important to regulate the amount of resorption that takes place, the motility of a given osteoclast and how much bone resorption occurs at a given location is also important. How long does an osteoclast spend in one place? What signals instruct it to move on? In cultures of osteoclasts on dentine, rodent-derived osteoclasts form characteristic resorption trails clearly demonstrating their motile nature. It has long been suggested that when the extracellular calcium concentration increases above a critical level, following release from the bone surface, resorption is inhibited as a consequence of disruption of cytoskeletal structure (Miyauchi *et al.* 1990) and that this may

involve preventing the release of phosphatases (reviewed in Zaidi *et al.* (2004)) or by induction of apoptosis (Lorget *et al.* 2000). *In vitro*, a rise in extracellular calcium results in a rise in cytosolic calcium that is released from intracellular stores and internalised across the plasma membrane, via activation of cell surface receptors that sense calcium. Although the mechanisms that sense this increase in extracellular calcium have not been confirmed, such receptors may include 1,4,5-trisphosphate receptors (IP3R) or ryanodine receptors, that also regulate release of calcium from internal stores, as well as an extracellular calcium-sensing receptor (Zaidi *et al.* 2004). The calcium release from intracellular stores is likely mediated by nitric oxide (NO), produced by osteoblasts and by osteoclasts themselves and a known mediator of osteoclast motility and/or apoptosis (Yaroslavskiy *et al.* 2005). Via cGMP synthesis NO activates protein kinase G that phosphorylates vasodilator-stimulated phosphoprotein (VASP), as part of the integrin complex and via SRC-family kinase activates the IP3R1 receptor on endoplasmic reticulum membranes. The subsequent increase in cytosolic calcium induces disassembly of the cytoskeleton (Miyauchi *et al.* 1990), possibly through activation of the calcium-dependent proteinase mu-calpain that is required for normal osteoclast motility (Marzia *et al.* 2006, Yaroslavskiy *et al.* 2007). This presumably enables the osteoclast to adopt a motile phenotype as it detaches from the bone surface. The calcium concentration surrounding the osteoclast would then be diluted, allowing the osteoclast to repolarise and assemble a new sealing zone. The matrix itself also plays a crucial role in the process of osteoclast detachment and reattachment since osteopontin, an extracellular bone matrix protein, has long been recognised as an autocrine factor to stimulate osteoclast migration via $\alpha_v\beta_3$ and CD44 (Chellaiiah & Hruska 2003) and TRAP may act to dephosphorylate osteopontin, and regulate osteoclast migration (Ek-Rylander & Andersson 2010).

Conclusion

Osteoclasts play a key role in the process of bone modelling and bone remodelling. The receptors and ligands essential for osteoclast formation, osteoclast fusion and osteoclast polarisation and resorption have been increasingly uncovered, aided by the discovery of critical genes involved in monogenic bone disorders and gene knockout technology. While receptor or ligand loss- or gain-of-function mutations, or indeed mutations in key components of their downstream signalling pathways, may lead to severe osteoclast phenotypes and hence severe bone disease such as osteopetrosis, more subtle differences in these pathways are likely to contribute to differences in bone mass in the general populations. Genome-wide association studies have uncovered critical roles for many of the proteins discussed in this review in postmenopausal osteoporosis and other bone diseases (Kiel *et al.* 2007, Rivadeneira *et al.* 2009, Albagha *et al.* 2010). In addition, pathway analysis has revealed further links between bone mass

and specific pathways such as autophagy (Zhang *et al.* 2010). We are some way off using this knowledge in a clinical setting, but our increasing understanding of the physiology of bone resorption and the aetiology of bone disease has been advanced substantially by these findings. We anticipate further advances as current studies are focusing specifically on the analysis of signalling pathways already known to be important in osteoclast biology, such as, for example, the RANK pathway and begin to analyse genetic variation in all genes involved in its downstream signalling (as shown in Fig. 1B), to determine their combined contribution to the development of bone disease. Overall, increased knowledge of osteoclast biology over the past decade has contributed to better understanding of bone biology and bone pathology and thereby identified a range of new targets to combat bone disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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