REVIEW ARTICLE

LS Holliday
DA Ostrov
TJ Wronski
C Dolce

Osteoclast polarization and orthodontic tooth movement

Authors' affiliations:
L.S. Holliday, C. Dolce, Department of Orthodontics, University of Florida College of Dentistry, Gainesville, Florida, USA
D.A. Ostrov, Department of Pathology, Immunology and Laboratory Science, University of Florida College of Medicine, Gainesville, Florida, USA
T.J. Wronski, Department of Physiological Sciences, University of Florida College of Veterinary Medicine, Gainesville, Florida, USA

Correspondence to:
Lexie Shannon Holliday
Department of Orthodontics
University of Florida College of Dentistry
Gainesville, FL 32610, USA
E-mail: SHOLLIDAY@dental.ufl.edu

Dates:
Accepted 4 February 2009

To cite this article:
Holliday LS, Ostrov DA, Wronski TJ, Dolce C: Osteoclast polarization and orthodontic tooth movement
Orthod Craniofac Res 2009;**12**:105–112

Structured Abstract

Authors – Holliday LS, Ostrov DA, Wronski TJ, Dolce C

Introduction – Osteoclasts polarize when they contact activation signals that are associated with bone. Polarization is required for bone resorption and involves highly specialized mechanisms that represent attractive targets for the development of osteoclast-specific therapeutic agents. One potential use of such agents is to block tooth movement in spatially discrete locations to provide orthodontic anchorage.

Materials and methods – Our group's research was directed toward the development of agents that inhibited the polarization of osteoclasts, and efforts were underway to develop means to experimentally modulate orthodontic tooth movement. We performed 'proof-in-principle' experiments demonstrating pharmacological blockades of orthodontic tooth movement using integrin and matrix metalloproteinase inhibitors in a rat model.

Results – We identified novel mechanisms underlying osteoclast bone resorption. Interactions between vacuolar H^+-ATPase and the microfilament cytoskeleton that were unique to osteoclasts were described and characterized. Our group is now seeking to make use of this new knowledge, coupled with an emerging technique, supercomputer-based molecular modeling for the rational development of novel, osteoclast-specific therapeutic agents.

Conclusion – Fresh insight into the molecular details of osteoclastic bone resorption provides new opportunities for identifying agents to selectively modulate osteoclast activity. Such agents may contribute to evolution of the practice of orthodontics.

Key words: computational chemistry; Elvax; microfilaments; orthodontic anchorage; vacuolar H^+-ATPase

Introduction

Orthodontic tooth movement (OTM) requires alterations in bone remodeling patterns in the alveolar bone underlying the tooth targeted for movement (1, 2). These changes involve increases in resorption on the pressure side of the tooth and increases in bone formation on the tension side. Several groups have reported either speeding or slowing OTM using agents that include receptor activator of nuclear factor kappa B-ligand (RANKL), osteoprotegerin, integrin inhibitors, matrix metalloproteinases inhibitors, and relaxin in animal models (3–8). Local inhibition of tooth movement could provide a convenient means to provide orthodontic anchorage.

Bone resorption by osteoclasts can be blocked either by preventing the differentiation of osteoclasts from precursors or by disabling the capacity of mature osteoclasts to resorb bone (Fig. 1). Whether one general approach or the other would be more useful for orthodontic applications is not known, but either might have unexpected adverse consequences (9, 10).

To identify novel inhibitors of OTM, we developed the concept of using small molecules or peptide modulators of osteoclast activity delivered in an inert, sustained-released package (polymers of ethylene vinyl acetate, ELVAX; Dupont, Wilmington, DE, USA), and have published two proof-of-principle reports (3, 4, 11–14). In our studies, we showed that either small peptides or small molecules were released over time in a biologically active form from ELVAX that was surgically implanted subgingivally, adjacent to the teeth of rats which were then subjected to orthodontic force, and that this treatment blocked OTM. The strategies of blocking either integrins or matrix metalloproteinases which we utilized to inhibit OTM are too likely to have collateral effects to be considered as practical elements of standard orthodontic practice (15, 16). Likewise, other agents used to block osteoclast activity, like the currently available bisphosphonates, have displayed adverse effects including oral osteonecrosis (17, 18).

A recent proof-in-principle study showed that injection of recombinant osteoprotegerin, an inhibitor of RANKL, could be utilized to block osteoclast formation, and provided orthodontic anchorage in a rat model (8). Whether osteoprotegerin or other molecules with similar functions, like Denosumab (19, 20) (a humanized monoclonal antibody that blocks RANKL signaling and is currently undergoing human clinical trials), can be used in orthodontic practice is not clear. It must also be considered that RANKL and osteoprotegerin are immune modulators (21). The dual roles of components of the RANKL signaling pathway and many other cytokine signaling pathways in immunological response and regulation of bone remodeling has led to the concept of osteoimmunology (22). This cross-talk raises concerns that inhibition of the RANKL pathway may reduce resistance to oral pathogens, an important consideration in orthodontics.

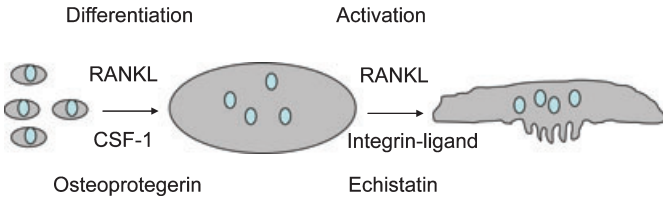

Fig. 1. Inhibition of orthodontic tooth movement has been achieved in animal models by blocking osteoclast differentation using osteoprotegerin (8) and by blocking osteoclast activation through the use of integrin (echistatin) and matrix metalloproteinase inhibitors (3, 4). It is not known which strategy will prove to be most useful in the orthodontic clinic. CSF, cerebrospinal fluid.

Here, our work demonstrating the importance of an interaction between the B subunit of the vacuolar H^+-ATPase (V-ATPase) and microfilaments in the polarization of osteoclasts and in bone resorption will be described. We believe this interaction represents an attractive novel target for agents that selectively block osteoclast bone resorption. We will then briefly discuss a strategy by which emerging computational chemistry techniques can be used in a rational approach for identifying small molecules that are useful as therapeutic agents (23–26). As such approaches become more sophisticated, it will become increasingly practical to translate basic science insight into advances in clinical practice. Because the expense of computational chemistry approaches to drug discovery are relatively low, such approaches may move drug discovery from the domain of big science to the small academic laboratory. This promises to bring many more minds, and with them fresh ideas, to the process of drug discovery.

The resorptive osteoclast: a highly specialized polar cell

Osteoclasts differentiate from multipotential hematopoetic precursors in response to stimulation by RANKL (21, 27, 28). In addition to RANKL, other requirements for osteoclast formation and for the activation of osteoclasts to resorb bone have been identified (29, 30).

For mature osteoclasts to resorb bone, activation signals must be present (31–34). Activation leads to profound reorganizations of the osteoclast's cytoskeleton resulting in the formation of structures known as actin rings on the apical surface of the osteoclast where it contacts bone (35–39). Actin rings are associated with very tight contacts between the plasma membrane and the bone, referred to as sealing zones, and are also linked to vesicular transport to and from ruffled membranes (40–45). Vital cargos of that trafficking in osteoclasts are V-ATPases (46, 47). Bounded by the

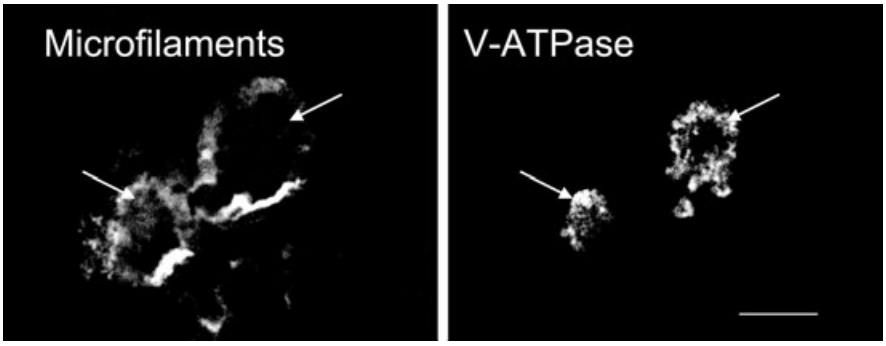

Fig. 2. Osteoclasts resorbing bone *in vitro* were fixed with formaldehyde and stained with fluorescein-tagged phalloidin to detect microfilaments and an anti-E subunit antibody visualized with a Texas Red-conjugated secondary antibody to detect V-ATPase, then visualized by confocal microscopy. The optical section shown is just at the bone surface. Note that the microfilaments are organized into structures called actin rings which surround the V-ATPase-rich ruffled membrane which is denoted with an arrow. The scale bar is equal to 20 μm.

actin rings, specialized domains of the plasma membrane are formed. Known as ruffled membranes (or ruffled borders) because they form brush border-like ruffles, they are the site from where proteolytic enzymes are dumped into resorption compartments (48–50). A very unusual feature of ruffled membranes is that they are packed with V-ATPases (46) (Fig. 2). Prior to activation, V-ATPases are stored in vesicular compartments in the cytoplasm (40, 51–53). These vesicles are associated with the actin cytoskeleton (40, 52, 54, 55). As part of the activation process, V-ATPase-rich vesicles are recruited to the nascent ruffled membrane and fuse with the plasma membrane to form the ruffled membrane. V-ATPases pump protons out of the cell into resorption compartments thereby lowering the pH to around 5.0 which is required for resorption (56). Only a few other specialized cell types have large reservoirs of plasma membrane V-ATPases (57, 58).

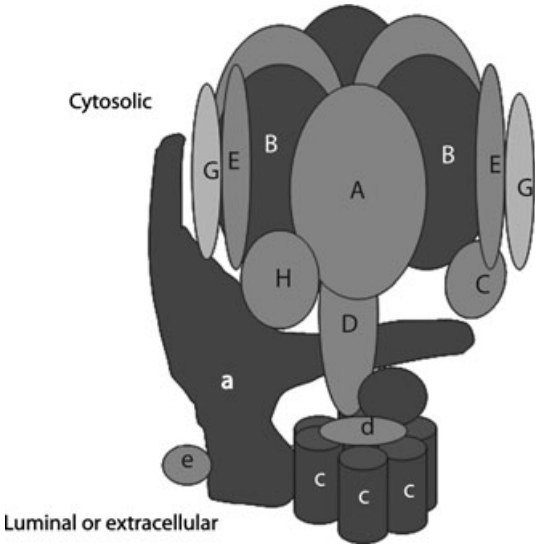

Fig. 3. V-ATPase are multisubunit enzymes composed of 11–13 protein subunits. The V-ATPase B subunit is the site of interaction with microfilaments. The two lines denote the position of the lipid bilayer.

V-ATPases

V-ATPases are composed of numerous subunits, some of which are present in multiple copies per holoenzyme (59–61), and are close relatives of the mitochondrial ATP synthase (Fig. 3). Like the synthase, V-ATPases are rotary motors. V-ATPases serve housekeeping roles in eukaryotic cells acidifying compartments of the endocytic pathway (61). Few cells express more than trace amounts of V-ATPase and a number of the subunits are present as isoforms. Characterization of tissue or cell type-specific isoforms has provided a measure of insight into the functions and cell biology of V-ATPases. (60–64). For example, there are four isoforms of subunit a in mammals (65). Subunits a1 and a2 are expressed ubiquitously and are found in endosomes and lysosomes. Subunit a3 is expressed at high levels in osteoclasts and pancreatic beta cells (62, 66). Subunit a4 is found in the kidney (65). Mutations in subunit a3 cause autosomal malignant osteopetrosis (67–69).

V-ATPase–actin interactions

In 1989, a seminal article in *Science* identified the V-ATPase as the enzyme responsible for acidification of resorption lacunae (46). This finding highlighted a dilemma; V-ATPases are vital enzymes in eukaryotic cells because they are required for acidification of

lysosomes, phagosomes, late endosomes, and compartments of the golgi. However, in most cells, V-ATPases appear to be forbidden from entry into the plasma membrane. How then could V-ATPases enter and pack subdomains of the plasma membrane of osteoclasts?

A number of the subunits of V-ATPase have multiple isoforms and mutations in some of the isoforms have been shown to be responsible for diseases (70, 71). Moreover, it is known that different isoforms of subunit a are targeted to differing cellular compartments when they are expressed in the same cell (51, 72). This has led to the idea that the isoforms have specific properties and activities that confer the ability to perform specialized functions to the V-ATPase into which they are incorporated. However, to date there is no clear mechanism to describe the mechanisms of isoform-specific regulation.

In 1999, we reported that V-ATPases in osteoclasts interact with microfilaments (40). The portion of V-ATPases bound to microfilaments changed with the activation state of the osteoclasts providing indirect evidence that the interaction is crucial for osteoclast function. The binding interaction between V-ATPase and microfilaments was high affinity (K_D = 55 nM). Direct visualization of V-ATPase bound to F-actin suggested that V-ATPase was binding at the top of the heterohexagon composed of subunits A and B making these two subunits the most likely candidates to mediate the interaction which we detected. Several lines of evidence then identified subunit B as an actin-binding protein, and we were able to reconstitute the interaction in the test tube using purified rabbit muscle actin and recombinant B subunit (73). Both the B2 isoform of subunit B, which is expressed at high levels in osteoclasts, and the B1 isoform, which is expressed at high levels in renal epithelial cells, bound F-actin equally well.

A profilin-like actin-binding site in subunit B

To identify the actin binding site, we made and expressed a series of overlapping recombinant fragments of both isoforms of subunit B (54). We identified a 44-amino acid region that was required for the actin-binding activity (amino acid 23–67 in mouse B1 and 29–73 in mouse B2). By direct inspection, our collaborator Michael R. Bubb (University of Florida College of Medicine) independently identified a region that shared sequence homology with the actin-binding site of the 'classic' actin-binding protein, mammalian profilin 1, within the 44 amino acid actin binding stretch (54). Peptides synthesized composed of the sequence of the profilin-like site and these were found to bind actin and compete with profilin for actin binding sites. By making use of the results of previous studies of the actin-binding site on profilin, we identified specific residues that we expected to be crucial for the actin-binding activity, and these predictions proved correct in our *in vitro* assays.

With our detailed knowledge of the actin-binding site in B subunit, we examined the sequences of B subunits in various organisms. We found that B subunits from organisms as evolutionarily diverse from humans as yeast, insects, and plants had sequences in the actin-binding region that would be consistent with the capacity to bind actin. We have confirmed that yeast B subunit binds actin (74), and another group showed the B subunit from *Manduca sexta* binds actin (75).

Actin-binding activity by B subunit is required for its transport to the ruffled membranes of osteoclasts

We found that adeno-associated virus (AAV), a viable viral vector for human gene therapy (76), transduced primary mouse osteoclasts efficiently (55). The AAV system allowed us to express exogenous mutant B1 which does not bind actin or wild type B1. Previous studies had shown that when B1 and B2 are expressed in the same cell, they do not co-assemble into the same V-ATPase (55) and osteoclasts normally express B2 not B1 (77). Therefore, we could follow virally expressed B1 separate from B2. Our studies showed clearly that actin-binding activity was required for transport of V-ATPases containing B1 to ruffled membranes; therefore the actin-binding activity was probably required for osteoclast bone resorption.

The structure of the actin-binding surface of B subunit

Our results suggested that an inhibitor of the actin-binding activity of B2 subunit would potentially be

useful as a therapeutic agent targeting osteoclast activity. Until recently, finding such an inhibitor would require the fortuitous identification of a natural product with a particular activity or the use of 'brute force' assays to identify lead molecules. Natural products like bafilomycin A1, an inhibitor of V-ATPase activity which is derived from *Streptomyces griseus* (78), are most often produced to be used in chemical warfare among species. Bafilomycin A1 is a potent toxin. It is unlikely that a osteoclast-specific agent would evolve in that context.

Brute force assays have proven useful but are expensive and are not well suited for the academic lab. Computational chemistry offers a potential alternate strategy that is relatively inexpensive. If the structure of a bioactive surface of interest is known through crystallography or by molecular modeling, docking programs can be used to perform a virtual screen for small molecules that are likely to interact with the surface. Although docking proteins are far from perfect, this technology is rapidly evolving with ever-increasing computing power and better understanding of protein structure.

We have begun screening for small molecule inhibitors of the V-ATPase–microfilament interaction. The first challenge was to identify structural pockets in subunit B that are suitable for interactions with small molecules. Although crystal structures of various actin-binding B subunits are not available, because of the sequence and structural similarity between B subunit and the alpha subunit of F-ATPase, which has been crystallized, high confidence structural models of B subunit isoforms could be proposed. We have utilized a comparative structural modeling approach to generate an atomic model of the murine B2 subunit. We have made use of the programs 3D-PSSM (Fig. 4) and Swiss Model. Our analysis identified the most probable structure of the protein sequence based on its relationships to the known similar structure of the alpha subunit of the F-ATPase.

We have begun to utilize modeling information derived from mammalian B2 subunit to identify ligands. Small molecules that binds the actin-binding site on subunit B2 specifically and sterically, inhibits its interaction with microfilaments and may prove to be a novel osteoclast inhibitor with favorable properties compared with other pharmaceuticals. We have to date (February 3, 2009) identified six small molecules based on the initial *in silico* screen that have the capacity to inhibit the interaction between B subunit and microfilaments in the test tube, and which inhibit osteoclast bone resorption in tissue culture. We will test the most promising inhibitor in a rat model of post-menopausal osteoporosis soon.

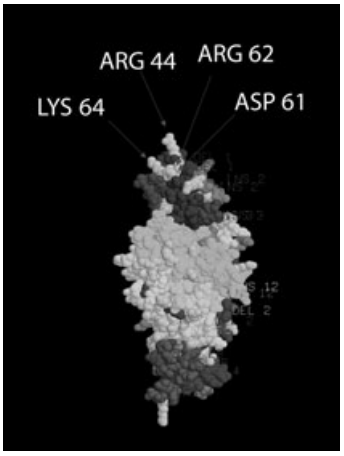

Fig. 4. Atomic level model of the B2 subunit of V-ATPase. This model was constructed using the 3D-PSSM which made use of known crystal structures of the α subunit of the ATP synthase, together with the sequence similarity between the B2 subunit and the α subunit. The subunit is positioned so that the top is the region furthest away from the lipid bilayer if it were incorporated into a proton pump. The residues noted are crucial candidiates for the actin binding activity of the subunit. The minimal site that supported full actin binding activity was amino acids 29–73, but the profilin-like domain, composed of amino acids 55–68, supported a lower level of actin binding activity (54).

Future prospects: from binding surfaces to pharmaceutical small molecules

Computational techniques that can 'dock' small molecules into the structures of protein targets are relatively new, and are a rapidly evolving approach for leveraging structural information of proteins for the identification of lead molecules in drug development. This may offer a path toward revolutionary advances in biomedical science. Although computational techniques are currently far from perfect, they now make it possible to narrow the number of small molecules to be screened so that it is manageable for many academic labs. Academic laboratory involvement is further encouraged by a large repository of small molecules maintained by the National Cancer Institute, which are available at nominal charge to academic researchers (http://dtp.nci.nih.gov/repositories.html).

For us, this new technology provides an opportunity to rationally attempt to move the basic science information that we have accumulated regarding interactions between V-ATPase and microfilaments in osteoclasts from the lab bench in the direction of the bedside and the orthodontist's chair. Our approach represents our particular adaptation of a general paradigm by which the labors of the basic scientist may bear fruits in the form of novel clinical tools. Computational techniques are developing rapidly but will also require greater cellular and molecular understanding of the involved cells and tissues and of the crucial protein structures that underlie the cellular mechanisms. It seems likely that during the twenty-first century, these new techniques will revolutionize the biomedical enterprise as the rapidly accumulating biological knowledge is tapped in ever more sophisticated ways for clinical use. One such avenue is in moving toward molecular orthodontics.

Clinical relevance

Orthodontic tooth movement could be augmented by inhibiting the movement of specific teeth by local administration of therapeutic agents. The immobilized teeth could be used to provide orthodontic anchorage. A rational approach to achieving this end is described based on understanding of the molecular underpinnings of osteoclast bone resorption using computer-based techniques to identify small molecules predicted to intervene in resorption in a novel way, and then using *in vitro* and *in vivo* models to test the local delivery and function of the small molecules that are identified.

References

1. Wise GE, King GJ. Mechanisms of tooth eruption and orthodontic tooth movement. *J Dent Res* 2008;87:414–34.
2. Krishnan V, Davidovitch Z. Cellular, molecular, and tissue-level reactions to orthodontic force. *Am J Orthod Dentofacial Orthop* 2006;129:469–32.
3. Dolce C, Vakani A, Archer L, Morris-Wiman JA, Holliday LS. Effects of echistatin and an RGD peptide on orthodontic tooth movement. *J Dent Res* 2003;82:682–6.
4. Holliday LS, Vakani A, Archer L, Dolce C. Effects of matrix metalloproteinase inhibitors on bone resorption and orthodontic tooth movement. *J Dent Res* 2003;82:687–91.
5. Kanzaki H, Chiba M, Takahashi I, Haruyama N, Nishimura M, Mitani H. Local OPG gene transfer to periodontal tissue inhibits orthodontic tooth movement. *J Dent Res* 2004;83:920–5.
6. Kanzaki H, Chiba M, Arai K, Takahashi N, Harayama M, Nishimura N et al. Local RANKL gene transfer to the periodontal tissue accelerates orthodontic tooth movement. *Gene Ther* 2006;13:678–85.
7. Stewart DR, Sherick P, Kramer S, Breining P. Use of relaxin in orthodontics. *Ann NY Acad Sci* 2005;1041:379–87.
8. Dunn MD, Park CH, Kostenuik PJ, Kapila S, Giannobile WV. Local delivery of osteoprotegerin inhibits mechanically mediated bone modeling in orthodontic tooth movement. *Bone* 2007;41:446–55.
9. Karsdal MA, Henriksen K, Sorensen MG, Gram J, Schaller S, Dziegiel MH et al. Acidification of the osteoclastic resorption compartment provides insight into the coupling of bone formation to bone resorption. *Am J Pathol* 2005;166:467–76.
10. Karsdal MA, Martin TJ, Bollerslev J, Christiansen C, Henriksen K. Are nonresorbing osteoclasts sources of bone anabolic activity? *J Bone Miner Res* 2007;22:487–94.
11. Burt HM, Jackson JK, Bains SK, Liggins RT, Oktaba AM, Arsenault AL et al. Controlled delivery of taxol from microspheres composed of a blend of ethylene-vinyl acetate copolymer and poly (D,L-lactic acid). *Cancer Lett* 1995;88:73–9.
12. Mitchell CA, Davies MJ, Grounds MD, McGeachie JK, Crawford GJ, Hong Y et al. Enhancement of neovascularization in regenerating skeletal muscle by the sustained release of erucamide from a polymer matrix. *J Biomater Appl* 1996;10:230–49.
13. Mitchell CA, McGeachie JK, Grounds MD. The exogenous administration of basic fibroblast growth factor to regenerating skeletal muscle in mice does not enhance the process of regeneration. *Growth Factors* 1996;13:37–55.
14. Langer R, Folkman J. Polymers for the sustained release of proteins and other macromolecules. *Nature* 1976;263:797–800.
15. Masarachia P, Yamamoto M, Leu CT, Rodan G, Duong L. Histomorphometric evidence for echistatin inhibition of bone resorption in mice with secondary hyperparathyroidism. *Endocrinology* 1998;139:1401–10.
16. Yamamoto M, Fisher JE, Gentile M, Seedor JG, Leu CT, Rodan SB et al. The integrin ligand echistatin prevents bone loss in ovariectomized mice and rats. *Endocrinology* 1998;139:1411–9.
17. Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D et al. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for bone and mineral research. *J Bone Miner Res* 2007;22:1479–91.
18. Bilezikian JP. Osteonecrosis of the jaw: do bisphosphonates pose a risk? *N Engl J Med* 2006;355:2278–81.
19. Hamdy NA. Denosumab: RANKL inhibition in the management of bone loss. *Drugs Today (Barc)* 2008;44:7–21.
20. McClung MR, Lewiecki EM, Cohen SB, Bolognese MA, Woodson GC, Moffett AH et al. Denosumab in postmenopausal women with low bone mineral density. *N Engl J Med* 2006;354:821–31.
21. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymphnode organogenesis. *Nature* 1999;397:315–23.
22. Walsh MC, Kim N, Kadono Y, Rho J, Lee SY, Lorenzo J et al. Osteoimmunology: interplay between the immune system and bone metabolism. *Annu Rev Immunol* 2006;24:33–63.

23. Morreale A, Iriepa I, Galvez E. The 5-HT(3) and nACh ionotropic receptors: a perspective from the computational chemistry point of view. *Curr Med Chem* 2002;9:99–125.
24. Kitchen DB, Decornez H, Furr JR, Bajorath J. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nat Rev Drug Discov* 2004;3:935–49.
25. Ricke DO, Wang S, Cai R, Cohen D. Genomic approaches to drug discovery. *Curr Opin Chem Biol* 2006;10:303–8.
26. Pozzan A. Molecular descriptors and methods for ligand based virtual high throughput screening in drug discovery. *Curr Pharm Des* 2006;12:2099–110.
27. Hofbauer LC, Kuhne CA, Viereck V. The OPG/RANKL/RANK system in metabolic bone diseases. *J Musculoskelet Neuronal Interact* 2004;4:268–75.
28. Kong YY, Penninger JM. Molecular control of bone remodeling and osteoporosis. *Exp Gerontol* 2000;35:947–56.
29. Novack DV, Teitelbaum SL. The osteoclast: friend or foe? *Annu Rev Pathol* 2008;3:457–84.
30. Teitelbaum SL. Osteoclasts: what do they do and how do they do it? *Am J Pathol* 2007;170:427–35.
31. Holliday LS, Welgus HG, Fliszar CJ, Veith GM, Jeffrey JJ, Gluck SL. Initiation of osteoclast bone resorption by interstitial collagenase. *J Biol Chem* 1997;272:22053–8.
32. Adams AE, Abu-Amer Y, Chappel J, Stueckle S, Ross FP, Teitelbaim SL et al. 1,25 dihydroxyvitamin D3 and dexamethasone induce the cyclooxygenase 1 gene in osteoclast-supporting stromal cells. *J Cell Biochem* 1999;74:587–95.
33. McHugh KP, Kitazawa S, Teitelbaum SL, Ross FP. Cloning and characterization of the murine beta(3) integrin gene promoter: identification of an interleukin-4 responsive element and regulation by STAT-6. *J Cell Biochem* 2001;81:320–32.
34. McHugh KP, Hodivala-Dilke K, Zheng MH, Namba N, Lam J, Novack D et al. Mice lacking beta 3 integrins are osteosclerotic because of dysfunctional osteoclasts. *J Clin Invest* 2000;105:433–40.
35. King GJ, Holtrop ME. Actin-like filaments in bone cells of cultured mouse calvaria as demonstrated by binding to heavy meromyosin. *J Cell Biol* 1975;66:445–51.
36. Lakkakorpi PT, Vaananen HK. Cytoskeletal changes in osteoclasts during the resorption cycle. *Microsc Res Tech* 1996;33:171–81.
37. Saltel F, Destaing O, Bard F, Eichert D, Jurdic P. Apatite-mediated actin dynamics in resorbing osteoclasts. *Mol Biol Cell* 2004;15:5231–41.
38. Jurdic P, Saltel F, Chabadel A, Destaing O. Podosome and sealing zone: specificity of the osteoclast model. *Eur J Cell Biol* 2006;85:195–202.
39. Destaing O, Saltel F, Geminard JC, Jurdic P, Bard F. Podosomes display actin turnover and dynamic self-organization in osteoclasts expressing actin-green fluorescent protein. *Mol Biol Cell* 2003;14:407–16.
40. Lee BS, Gluck SL, Holliday LS. Interaction between vacuolar H(+)-ATPase and microfilaments during osteoclast activation. *J Biol Chem* 1999;274:29164–71.
41. Holliday LS, Bubb MR, Jiang J, Hurst IR, Zuo J. Interactions between vacuolar H+-ATPases and microfilaments in osteoclasts. *J Bioenerg Biomembr* 2005;37:419–23.
42. Moreau V, Tatin F, Varon C, Genot E. Actin can reorganize into podosomes in aortic endothelial cells, a process controlled by Cdc42 and RhoA. *Mol Cell Biol* 2003;23:6809–22.
43. Ochoa GC, Slepnev VI, Neff L, Ringstad N, Takei K, Daniell L et al. A functional link between dynamin and the actin cytoskeleton at podosomes. *J Cell Biol* 2000;150:377–89.
44. Linder S, Aepfelbacher M. Podosomes: adhesion hot-spots of invasive cells. *Trends Cell Biol* 2003;13:376–85.
45. McNiven MA, Baldassarre M, Buccione R. The role of dynamin in the assembly and function of podosomes and invadopodia. *Front Biosci* 2004;9:1944–53.
46. Blair HC, Teitelbaum SL, Ghiselli R, Gluck S. Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* 1989;245:855–7.
47. Vaananen HK, Karhukorpi EK, Sundquist K, Wallmark B, Roininen I, Hentunen TT et al. Evidence for the presence of a proton pump of the vacuolar H(+)-ATPase type in the ruffled borders of osteoclasts. *J Cell Biol* 1990;111:1305–11.
48. Vaananen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. *J Cell Sci* 2000;113:377–81.
49. Bromme D, Okamoto K, Wang BB, Biroc S. Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. Functional expression of human cathepsin O2 in *Spodoptera frugiperda* and characterization of the enzyme. *J Biol Chem* 1996;271:2126–32.
50. Gelb BD, Shi GP, Chapman HA, Desnick RJ. Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* 1996;273:1236–8.
51. Toyomura T, Murata Y, Yamamoto A, Oka T, Sun-Wada GH, Wada Y et al. From lysosomes to the plasma membrane: localization of vacuolar type H+-ATPase with the a3 isoform during osteoclast differentiation. *J Biol Chem* 2003;278:22023–30.
52. Nakamura I, Takahashi N, Udagawa N, Morimura Y, Kurokawa T, Jimi E et al. Lack of vacuolar proton ATPase association with the cytoskeleton in osteoclasts of osteosclerotic (oc/oc) mice. *FEBS Lett* 1997;401:207–12.
53. Nakamura I, Sasaki T, Tanaka S, Takahashi N, Jimi E, Kurokawa T et al. Phosphatidylinositol-3 kinase is involved in ruffled border formation in osteoclasts. *J Cell Physiol* 1997;172:230–9.
54. Chen SH, Bubb MR, Yarmola EG, Zuo J, Jiang J, Lee BS et al. Vacuolar H+-ATPase binding to microfilaments: regulation in response to phosphatidylinositol 3-kinase activity and detailed characterization of the actin-binding site in subunit B. *J Biol Chem* 2004;279:7988–98.
55. Zuo J, Jiang J, Chen SH, Vergara S, Gong Y, Xue J et al. Actin binding activity of subunit b of vacuolar H(+)-ATPase is involved in its targeting to ruffled membranes of osteoclasts. *J Bone Miner Res* 2006;21:714–21.
56. Baron R, Neff L, Louvard D, Courtoy PJ. Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. *J Cell Biol* 1985;101:2210–22.
57. Wagner CA, Finberg KE, Breton S, Marshansky V, Brown D, Geibel JP. Renal vacuolar H+-ATPase. *Physiol Rev* 2004;84:1263–314.
58. Breton S, Smith PJ, Lui B, Brown D. Acidification of the male reproductive tract by a proton pumping (H+)-ATPase. *Nat Med* 1996;2:470–2.
59. Gluck SL, Lee BS, Wang SP, Underhill D, Nemoto J, Holliday LS. Plasma membrane V-ATPases in proton-transporting cells of the mammalian kidney and osteoclast. *Acta Physiol Scand Suppl* 1998;643:203–12.

60. Cipriano DJ, Wang Y, Bond S, Hinton A, Jefferies KC, Qi J et al. Structure and regulation of the vacuolar ATPases. *Biochim Biophys Acta* 2008;1777:599–604.
61. Forgac M. Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nat Rev Mol Cell Biol* 2007;8:917–29.
62. Sun-Wada GH, Toyomura T, Murata Y, Yamamoto A, Futai M, Wada Y. The a3 isoform of V-ATPase regulates insulin secretion from pancreatic beta cells. *J Cell Sci* 2006;119:4531–40.
63. Sun-Wada GH, Yoshimizu T, Imai-Senga Y, Wada Y, Futai M. Diversity of mouse proton-translocating ATPase: presence of multiple isoforms of the C, d and G subunits. *Gene* 2003;302:147–53.
64. Smith AN, Borthwick KJ, Karet FE. Molecular cloning and characterization of novel tissue-specific isoforms of the human vacuolar H(+)-ATPase C, G and d subunits, and their evaluation in autosomal recessive distal renal tubular acidosis. *Gene* 2002;297:169–77.
65. Smith AN, Finberg KE, Wagner CA, Lifton RP, Devonald MA, Su Y et al. Molecular cloning and characterization of Atp6n1b: a novel fourth murine vacuolar H+-ATPase a-subunit gene. *J Biol Chem* 2001;276:42382–8.
66. Manolson MF, Yu H, Chen W, Yao Y, Li K, Lees RL et al. The a3 isoform of the 100-kDa V-ATPase subunit is highly but differentially expressed in large (> or = 10 nuclei) and small (<or = nuclei) osteoclasts. *J Biol Chem* 2003;278:49271–8.
67. Li YP, Chen W, Liang Y, Li E, Stashenko P. Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. *Nat Genet* 1999;23:447–51.
68. Frattini A, Orchard PJ, Sobacchi C, Giliani S, Abinun M, Mattsson JP et al. Defects in TCIRG1 subunit of the vacuolar proton pump are responsible for a subset of human autosomal recessive osteopetrosis. *Nat Genet* 2000;25:343–6.
69. Ogbureke KUE, Zhao QX, Li YP. Human osteopetroses and the osteoclast V-H+-atpase acidification system. *Front Biosci* 2005;10:2940–U117.
70. Karet FE. Physiological and metabolic implications of V-ATPase isoforms in the kidney. *J Bioenerg Biomembr* 2005;37:425–9.
71. Qi J, Wang Y, Forgac M. The vacuolar H(+)-ATPase: subunit arrangement and in vivo regulation. *J Bioenerg Biomembr* 2007;39:423–6.
72. Perzov N, Padler-Karavani V, Nelson H, Nelson N. Characterization of yeast V-ATPase mutants lacking Vph1p or Stv1p and the effect on endocytosis. *J Exp Biol* 2002;205:1209–19.
73. Holliday LS, Lu M, Lee BS, Nelson RD, Solivan S, Zhang L et al. The amino-terminal domain of the B subunit of vacuolar H+-ATPase contains a filamentous actin binding site. *J Biol Chem* 2000;275:32331–7.
74. Zuo J, Vergara S, Kohno S, Holliday LS. Biochemical and functional characterization of the actin-binding activity of the B subunit of yeast vacuolar H+-ATPase. *J Exp Biol* 2008;211:1102–8.
75. Vitavska O, Wieczorek H, Merzendorfer H. A novel role for subunit C in mediating binding of the H+-V-ATPase to the actin cytoskeleton. *J Biol Chem* 2003;278:18499–505.
76. Hauswirth WW, Lewin AS, Zolotukhin S, Muzyczka N. Production and purification of recombinant adeno-associated virus. *Methods Enzymol* 2000;316:743–61.
77. Lee BS, Holliday LS, Ojikutu B, Krits I, Gluck SL. Osteoclasts express the B2 isoform of vacuolar H(+)-ATPase intracellularly and on their plasma membranes. *Am J Physiol* 1996;270:C382–8.
78. Werner G, Hagenmaier H, Drautz H, Baumgartner A, Zahner H. Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity. *J Antibiot (Tokyo)* 1984;37:110–7.