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REVIEW ARTICLE

Advances in osteoclast biology resulting from the study of osteopetrotic mutations

T. Segovia-Silvestre · A. V. Neutzsky-Wulff · M. G. Sorensen · C. Christiansen · J. Bollerslev · M. A. Karsdal · K. Henriksen

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Abstract Osteopetrosis is the result of mutations affecting osteoclast function. Careful analyses of osteopetrosis have provided instrumental information on bone remodeling, including the coupling of bone formation to bone resorption. Based on a range of novel genetic mutations and the resulting osteoclast phenotypes, we discuss how osteopetrosis models have clarified the function of the coupling of bone formation to bone resorption, and the pivotal role of the osteoclast and their function in this phenomenon. We highlight the distinct possibility that osteoclast activities can be divided into two separate avenues: bone resorption and control of bone formation.

Introduction

Bone is a highly specialized tissue, which in combination with cartilage forms the skeleton. Bones has at least three functions: (1) mechanical, as support for muscle attachment for locomotion, (2) protective, as shields for the vital organs and the bone marrow, (3) metabolic, as a storage facility for ions, mainly calcium and phosphate, and for growth factors and cytokines (Baron 2005).

Bone is divided into an inorganic and an organic phase. The organic phase mainly consists of type I collagen, which is about 90% of the proteins in bone (Baron 2005). The type I collagen molecules are constructed as triple helices, which are oriented into fibers thereby providing maximum tensile strength. Molecular cross-links between the triple helices provide additional strength (Seeman and Delmas 2006). In addition to type I collagen numerous non-collagenous proteins are present in bone (Baron, 2005), of which osteopontin, osteocalcin, osteonectin are known to play important roles (Robey and Boskey 2006).

The inorganic phase of the bones provides the rigidity and load bearing required for strength, and the calcium and phosphate ions are present as hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], which forms extremely small crystals, which are easily soluble and thereby compatible with the ion reservoir function of bone (Robey and Boskey 2006).

Bone remodelling is an essential process, which is involved in maintaining both bone quality and strength, as well as calcium homeostasis (Seeman and Delmas 2006). Two kinds of remodeling have been suggested, each with a different purpose (Burr 2002; Parfitt 2002). The first type is stochastic, and it is primarily dedicated to maintenance of the calcium homeostasis, and it is under hormonal control (Noble 2003). The second type is targeted toward removal of microdamage, and thus essential for the mechanical properties of the skeleton (Burr 2002; Parfitt 2002).

In healthy adults targeted remodeling is a continuous physiological process, and initially bone formation was shown to always follow bone resorption leading to full replenishment of removed bone matrix with newly synthesized bone (Hattner et al. 1965; Takahashi et al. 1964). Remodeling, thus, is understood as a cycle of bone resorption

T. Segovia-Silvestre · A. V. Neutzsky-Wulff · M. G. Sorensen · M. A. Karsdal · K. Henriksen (✉)
Nordic Bioscience A/S, Herlev Hovedgade 207, 2730 Herlev, Denmark
e-mail: kh@nordicbioscience.com

C. Christiansen
Center for Clinical and Basic Research, CCBR, Ballerup, Denmark

J. Bollerslev
Section of Endocrinology, Department of Medicine, Rikshospitalet Medical Clinic, University of Oslo, Oslo, Norway

by osteoclasts and formation of new bone by osteoblasts, in which formation is tightly coupled to resorption, hence the term "the coupling mechanism" (Martin 1993; Martin and Sims 2005).

The remodeling cycle starts at specific sites (Fig. 1). The damage caused to osteocytes by an occurrence of microdamage appears to initiate the recruitment of osteoclast precursors, osteoclastogenesis and resorption of the mineralized bone matrix (Noble et al. 2003; Roodman 1999; Tatsumi et al. 2007). They subsequently die by apoptosis, and the reversal phase is initiated leading to the formation of a cement line. The cement line marks the extent of bone resorption and aggregates together old and new bone (Baron 2005; Dodds et al. 1995; Everts et al. 2002; Mulari et al. 2004).

Bone formation starts when the vacated resorption pits are populated by mononuclear cells (Hattner et al. 1965; Huffer 1988; Mulari et al. 2004; Takahashi et al. 1964; Tran et al. 1982). These non-identified cells remove non-resorbed remains from the bone, such as undigested collagen fibers, and prepare the area for osteoblasts to form new bone (Everts et al. 2002). A complete remodeling cycle lasts 4 months, comprising 20–30 days of resorption and 3 months of bone formation (Martin and Seeman 2007).

The coupling of bone formation to bone resorption during remodeling is a major topic in bone research since imbalances in this mechanism lead to pathological situations, such as osteoporosis or osteopetrosis (Karsdal et al. 2007; Rodan and Martin 2000).

The osteoclast

Osteoclasts are large multinuclear cells unique in their ability to resorb the mineralized bone matrix (Roodman 1999). They originate from hematopoietic stem cells (HSCs) (Marks and Walker 1981), which differentiate into bone resorbing osteoclasts through a series of steps involving commitment of HSCs into the monocyte/macrophage lineage, proliferation of pre-osteoclasts, differentiation into osteoclasts, and cell polarization enabling resorptive activity (Roodman 2006).

Osteoclastogenesis is controlled by two cytokines and their corresponding receptors. Macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κ B Ligand (RANKL) are essential for osteoclast lifespan and function (Boyle et al. 2003; Wiktor-Jedrzejczak et al. 1990). c-fms and receptor activator of nuclear factor κ B (RANK) are the receptors for M-CSF and RANKL (Arai et al. 1999; Lacey et al. 1998; Li et al. 2000). Loss of any of these molecules leads to complete absence of osteoclasts, illustrating their importance (Boyle et al. 2003; Wiktor-Jedrzejczak et al. 1990). Osteoprotegerin (OPG) is a soluble receptor for RANKL and it is involved in the

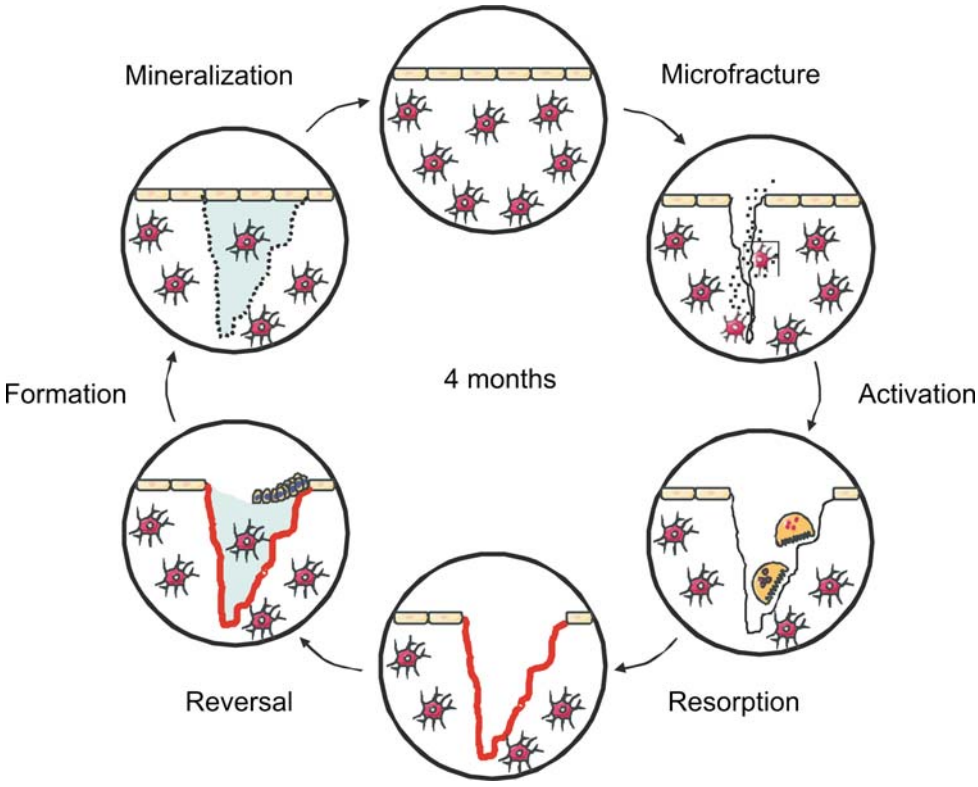

Fig. 1 Remodeling of bone matrix. Microcracks likely initiate remodeling through osteocyte apoptosis, which leads to bone resorption by the osteoclasts. After resorption the osteoclasts die by apoptosis, and bone formation is initiated leading to complete replenishment of the removed bone

regulation of both the number and the activity of the osteoclasts (Simonet et al. 1997; Yasuda et al. 1998), and is crucial for the maintenance of a healthy skeleton (Eghbali-Fatourechi et al. 2003).

Osteoclastogenesis requires a group of molecules, which are summarized in Fig. 2, but not discussed further. After initiation of differentiation, cells reach the pre-osteoclast stage, during which expression of tartrate resistant acid phosphatase (TRACP) can be observed (Roodman 2006). The next step in osteoclast maturation is correct assembly of the cytoskeleton and polarization (Vaananen and Horton 1995), eventually leading to tight attachment and the formation of a sealing zone which is visualized as an F-actin ring (Roodman 1999). The hallmark of a resorbing osteoclast is the formation of the intensely convoluted ruffled border inside the sealing zone (Fig. 3) (Roodman 1999). Mature osteoclasts are also characterized by high expression of a series of osteoclast markers, of which TRACP, Matrix Metallo Proteinase 9 (MMP-9), cathepsin K, Carbonic Anhydrase II (CAII), the a3 subunit of the V-ATPase, the chloride channel 7 (ClC-7), and osteopetrosis-associated TransMembrane Protein 1 (OSTM1) are the most prominent (Chalhoub et al. 2003; Findlay and Martin 1997; Frattini et al. 2000; Gelb et al. 1996; Hayman et al. 1996; Kornak et al. 2001; Sly et al. 1983; Vu et al. 1998).

Inside the sealing zone, bone resorption is induced by active secretion of protons through a specialized vacuolar type ATPase (V-ATPase), containing the a3 subunit (Frattini et al. 2000; Kornak et al. 2000; Li et al. 1999; Scimeca et al. 2000), and passive transport of chloride through the chloride channel ClC-7 (Fig. 4) (Blair et al. 1989; Blair et al. 1991; Henriksen et al. 2004; Karsdal et al. 2005; Kornak et al. 2001). This secretion of hydrochloric acid lowers the pH to approximately 4.5, leading to dissolution of the inorganic matrix of bone (Baron et al. 1985).

To generate the required levels of H^+ and Cl^- the osteoclasts utilize carbonic anhydrase II (CAII) that catalyzes conversion of CO_2 and H_2O into H_2CO_3, which in turn ionizes into H^+ and HCO_3^- (Sly et al. 1983; Tolar et al. 2004). The HCO_3^- ions are then exchanged for Cl^- through the

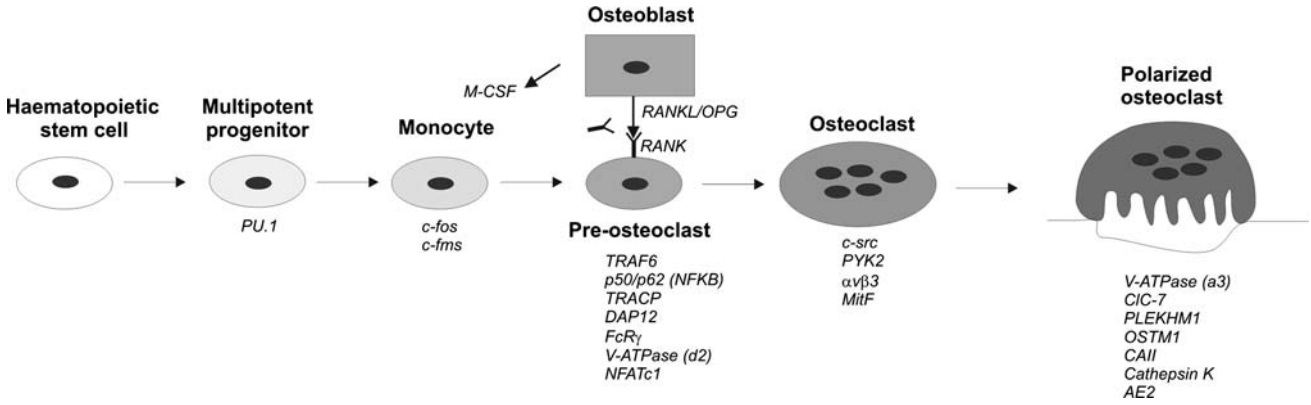

Fig. 2 Osteoclast differentiation. In the presence of RANKL and M-CSF the osteoclasts develop through a series of steps, involving fusion of the precursors, maturation and finally activation of bone resorption

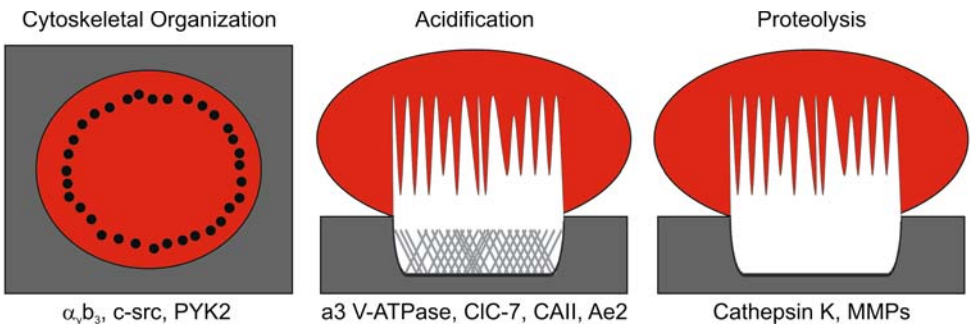

Fig. 3 Cytoskeletal reorganization Schematic illustration of the three processes required for bone resorption. The osteoclasts are *red*, and the bone is *gray*. Cytoskeletal organization is mediated by the $\alpha_v\beta 3$ integrin, c-src and PYK2 and leads to the formation of an actin ring (indicated by the *black dots*). Acidification of the resorption lacuna is mediated directly by the a3 V-ATPase and ClC-7, and indirectly by CAII and AE2, and leads to dissolution of the inorganic matrix of the bones, leaving demineralized collagen fibers in the resorption pit (*gray lines*). Proteolysis removes the collagen fibers in the resorption pits thereby preparing the pits for formation, and is mainly mediated by cathepsin K, although the MMPs also participate under some circumstances

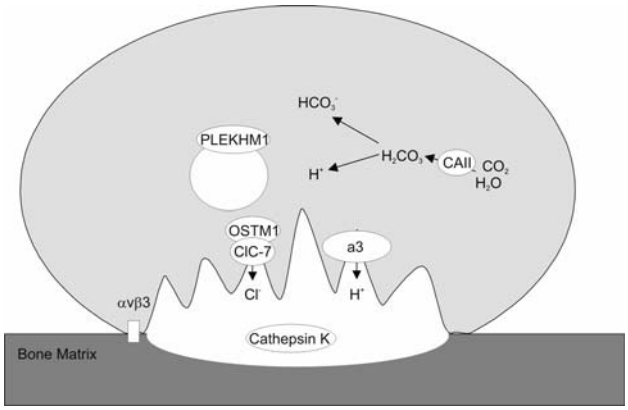

Fig. 4 Bone resorption. The mature osteoclasts resorb bone via secretion of hydrochloric acid through the specialized V-ATPase and the chloride channel ClC-7. Also required to perform resorption are carbonic anhydrase II (CAII), anion exchanger (AE2), $\alpha_v\beta_3$ integrin, PLEKHM1, OSTM1 and cathepsin K

basolaterally located Anion Exchanger 2 (AE2) (Jansen et al. 2006; Teti et al. 1989), providing the Cl^- ions required for the acidification occurring in the resorption lacuna.

Cleavage of the type I collagen fibers is mainly mediated by the cysteine proteinase cathepsin K, which is active at low pH (Bossard et al. 1996; Gowen et al. 1999; Nishi et al. 1999; Saftig et al. 1998), and performs almost complete removal of the type I collagen fibers (Everts et al. 2002). The MMPs are also involved in the degradation of the organic matrix of the bones; however, their exact role is still under investigation (Henriksen et al. 2006).

The resorbed material is removed from the resorption pit by transcytosis through the osteoclast (Nesbitt and Horton 1997; Salo et al. 1997). After having finished resorption, the osteoclasts will either perform a novel round of resorption or die (Roodman 1999).

In summary, osteoclasts are highly specialized cells dedicated to the dissolution of the inorganic matrix and the degradation of the organic matrix of the bones.

The osteoblast

The osteoblasts arise through a series of steps, involving expansion of their precursors called osteoprogenitors, differentiation into preosteoblasts, further differentiation into mature bone forming osteoblasts (Ducy et al. 2000; Manolagas 2000).

The differentiation of the mesenchymal stem cells (MSCs) into bone forming osteoblasts is controlled by a complex interplay between a series of cytokines, hormones and their corresponding receptors, of which the bone morphogenetic proteins (BMPs), transforming growth factor β (TGFβ), Insulin-like growth factors (IGFs), Wnts and their receptor low-density lipoprotein receptor-related protein 5 (LRP5), and parathyroid hormone (PTH) all use extensive signal transduction machinery to exert essential functions at different levels of osteoblastogenesis (Cohen 2006; Janssens et al. 2005; Koay and Brown 2005; Rosen 2003; Zou et al. 2006). Furthermore, the well-known transcription factor in the osteoblasts RUNX2 (Runt related transcription factor 2), also known as cbfa1, is required for commitment of the MSCs into the osteoblast lineage (Otto et al. 1997; Xiao et al. 2004). The transcription factor osterix, which is downstream of RUNX2, is essential for differentiation from preosteoblasts into mature osteoblasts (Nakashima et al. 2002).

Mature bone forming osteoblasts are never found alone; they always exist in small bone forming clusters, which consist of a heterogeneous mix of bone forming osteoblasts and bone lining cells (Aubin 2001). The osteoblasts are cuboidal in shape and are characterized by having a large nucleus, extensive Golgi and endoplasmatic reticulum, which all indicate a high level of protein synthesis (Aubin et al. 2006). The bone-forming osteoblasts express high levels of alkaline phosphatase activity (ALP) and secrete type I collagen onto bone surfaces forming new unmineralized bone matrix called osteoid (Aubin et al. 2006). After having laid down the osteoid, the osteoblasts mineralize the newly formed bone matrix by secretion of vesicles containing sufficient concentrations of calcium and phosphate to allow crystal formation (Aubin 2001; Manolagas 2000). In addition to the secretion of type I collagen, the osteoblasts also synthesize other non-collagenous matrix molecules, such as osteocalcin, osteopontin, osteonectin, and bone sialoprotein, which are required for correct mineralization of the bone matrix (Aubin et al. 2006).

After having completed bone formation the osteoblasts can undergo one of three potentials fates: (1) develop into bone lining cells (approximately 5%), (2) develop into osteocytes (approximately 25%), and (3) die by apoptosis (approximately 70%) (Manolagas 2000).

Osteopetrosis

Osteopetrosis is a heterogeneous group of rare genetic disorders characterized by increased skeletal mass due to defective osteoclast function (Tolar et al. 2004). It was first described by Albers-Schönberg a century ago (Albers-Schönberg 1904).

The diseases are classified into three severities: (1) Autosomal recessive osteopetrosis (ARO), or malignant infantile osteopetrosis, which has an incidence of 1:200,000–1:300,000 (Balemans et al. 2005). ARO often results in death before the age of four due to severe hematological defects (Balemans et al. 2005). (2) Intermediate autosomal

recessive osteopetrosis (IARO) shows many of the same characteristics as ARO, but has a longer life expectancy (Balemans et al. 2005; Tolar et al, 2004). (3) Benign, autosomal dominant osteopetrosis (ADO) are subdivided into type I and type II (ADOI and ADOII), which have a combined incidence of between 1:100,000 and 1:500,000 cases (Benichou et al. 2000). ADOI is caused by a mutation in the *LRP5* gene, and only indirectly affects osteoclast function (Boyden et al. 2002). Since recent studies have shown that ADOI is a high bone mass disorder caused by activation of the osteoblasts it will not discussed any further (Del Fattore et al. 2008a; Henriksen et al. 2005; Johnson et al. 2004; Tolar et al. 2004; Waguespack et al. 2007). ADOII is a disease, which in most cases, is caused by mutations in the *CLCN7* gene (Del Fattore et al. 2008a), and it is described in detail later. Pycnodysostosis (also known a Toulouse–Lautrec syndrome) is very rare disease, but it shares some radiological findings with osteopetrosis (Helfrich 2003).

ARO is normally diagnosed soon after birth or within the first years of life, whereas benign osteopetrosis often is diagnosed by coincidental radiography (Balemans et al. 2005; Bollerslev, 1989).

The typical radiological findings of all the forms of osteopetrosis, albeit with varying degrees of severity are: diffuse sclerosis of the spine and long bones, metaphyseal widening of the long bones, a "Rugger jersey" spine and a typical "bone-in-bone" appearance in phalanges, long bones and pelvic bones (Balemans et al. 2005; Taranta et al. 2003). The sclerotic bones are brittle and prone to fractures. Other clinical findings include severe anemia, hepatosplenomegaly, and pancytopenia, thickening of the skull, and osteomyelitis, all of which manifest themselves more or less depending on the severity of the osteopetrosis and the genetic background (Balemans et al. 2005; Frattini et al. 2003; Gerritsen et al. 1994b; Helfrich 2003; Tolar et al. 2004). Hearing loss and visual impairment are often associated with some forms of osteopetrosis and in some cases these effects are caused by cranial nerve compression, due to overgrowth of bone (Bollerslev et al. 1988; Gerritsen et al. 1994b; Thompson et al. 1998). If left untreated ARO usually is fatal within the first decade (Gerritsen et al. 1994a), and the only currently available treatment for ARO is bone marrow transplantation, which only succeeds in ~50% of the cases, likely due to irreversible damage occurring before transplantation (Driessen et al. 2003; Gerritsen et al. 1994a).

Mutations in ten human osteoclast proteins have so far been shown to lead to osteopetrosis or osteopetrosis-like phenotypes (Table 1): a3 V-ATPase (ARO), ClC-7 (ARO, IARO, ADOII), OSTM1 (ARO), CAII (IARO), PLEKHM1 (IARO), RANKL (ARO), RANK (ARO), cathepsin K (Pycnodysostosis), NEMO (not well-described) and the β3 integrin (different severities) (Table 1), and they will be described in the following sections. In this review mouse models will mainly be used to complement the original findings in humans.

The a3 subunit of the V-ATPase

The a3 subunit of the osteoclast vacuolar type H+-ATPase (V-ATPase) is a putative 7 transmembrane domain protein belonging to the V-ATPase 116 kDa subunit family. The a3 subunit is a core protein in the multimeric structure of the osteoclastic V-ATPase (Ogbureke et al. 2005; Xu et al. 2007).

The a3 subunit is highly expressed in mature osteoclasts, where it is localized at the ruffled border (Frattini et al. 2000; Manolson et al. 2003), and much of the current knowledge about this protein is from the study of patients and animal models, such as the oc/oc mice, bearing *TCIRG1* mutations (Frattini et al. 2000; Heaney et al. 1998; Kornak et al. 2000; Li et al. 1999; Sobacchi et al. 2001; Taranta et al. 2003). The a3 subunit is essential for the osteoclasts ability to acidify the resorption lacunae (Li et al. 1999). Recessive mutations or compound heterozygozity within the *TCIRG1* gene are the cause of approximately 50% of all ARO cases (Balemans et al. 2005; Kornak et al. 2000; Sobacchi et al. 2001; Taranta et al. 2003).

A characteristic finding in osteopetrotic bone biopsies is the presence of primary spongiosa and remnants of mineralized cartilage within the bones caused by loss of osteoclast function (Helfrich et al. 2007; Helfrich 2003; Tolar et al. 2004). A very recent study showed a high osteoid volume in these patients and *oc/oc* mice, a finding which was caused by defective gastric acid secretion, due to the *TCIRG1* mutations, and the reduced gastric acid levels were shown to lead to hypocalcemia causing the increased osteoid volume (Schinke et al. 2008).

In bone biopsies from patients harboring *TCIRG1* mutations the number of non-resorbing osteoclasts is higher than the number of osteoclasts found in healthy individuals (Flanagan et al. 2000; Frattini et al. 2000; Taranta et al. 2003). The osteoclasts in ARO patients are comparatively larger in size and have high numbers of nuclei, but show normal polarity and contact with bone (Taranta et al. 2003), and appear to show some residual bone resorption activity in vivo (Del Fattore et al. 2006; Taranta et al. 2003). In vitro studies of osteoclasts with loss of function mutations in the a3 subunit have shown that although the osteoclasts develop normally, their capacity to resorb bone is greatly diminished (Del Fattore et al. 2006; Taranta et al. 2003). This is due to a decreased potential for secretion of acid (Blair et al. 2004), a phenotypic trait also present in *Atp6i* deficient mice (Li et al. 1999). In contrast, studies of bone

Table 1 Summary of all known proteins leading to osteopetrotic phenotypes, the nomenclature and their function, and references to the articles describing the mutations for the first time

Gene	Name	Function	NCBI Gene	OMIM	Phenotype	References
TCIRG1	T-cell, immune regulator 1, ATPase, H + transporting, lysosomal V0 subunit A3	Acidification of the resorption lacuna	10312	259700, 604592	ARO	Frattini et al. 2000 Kornak et al. 2000
CLCN7	Chloride channel 7	Acidification of the resorption lacuna	1186	259700, 166600	ARO/IARO/ADOII	Kornak et al. 2001 Cleiren et al. 2001 Campos-Xavier et al. 2003 Frattini et al. 2003
OSTM1	Osteopetrosis associated transmembrane protein 1	β-subunit for ClC-7	28962	259700, 607649	ARO	Chalhoub et al. 2003
CTSK	Cathepsin K	Collagen degradation	1513	265800, 601105	PKND	Gelb et al. 1996
CA2	Carbonic anhydrase II	Intracellular acidification	760	259730	IARO	Sly et al. 1983
PLEKHM1	Pleckstrin homology domain containing, family M (with RUN domain) member 1	Vesicular trafficking	303584	259700, 611497	IARO	Van Wesenbeeck et al. 2007
TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11 (RANKL)	Osteoclastogenesis, resorption, survival	8600	602642	ARO	Sobacchi et al. 2007
TNFRSF11A	Tumor necrosis factor (ligand) superfamily, member 11 A (RANK)	Osteoclastogenesis, resorption, survival	8792	603499	ARO	Guerrini et al. 2008
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (NEMO)	Not clear yet	8517	300301, 300291	Very few cases	Dupuis-Girod et al. 2002
ITGB3	β3 Integrin	Cytoskeletal organization	3690	173470	Heterogeneous	Yarali et al. 2003

formation indices have shown that the markers of bone formation are increased, although there is still some controversy regarding the markers (Del Fattore et al. 2006; Taranta et al. 2003). Finally, the number of osteoblasts was shown to be directly correlated to the number of non-resorbing osteoclasts (Del Fattore et al. 2006).

Thus, these data are suggestive of ongoing bone formation in the absence of resorption, indicating that the coupling of bone formation to bone resorption has been distorted.

Chloride channel 7 (ClC-7)

The chloride channel 7 (ClC-7) is a multipass membrane protein that functions as a Cl^-/H^+ exchanger regulated by voltage-gating (Graves et al. 2008), the most recently discovered member of the mammalian CLC gene family (Brandt and Jentsch 1995). In osteoclasts ClC-7 is localized in the lysosomes and the ruffled border (Kornak et al. 2001; Schaller et al. 2004). *CLCN7* mutations in osteopetrosis patients and *Clcn7−/−* mice have provided evidence supporting that the main role for ClC-7 is in dissolution of the inorganic bone matrix (Henriksen et al. 2004; Kornak et al. 2001), where it provides the chloride conductance required for an efficient proton pumping by the V-ATPase (Kornak et al. 2001), and thus acid efflux.

Mutations in ClC-7 give rise to osteopetrosis with varying degrees of severity ranging from asymptomatic, to relatively mild symptoms in ADOII patients, to the very severe phenotype of ARO (Cleiren et al. 2001; Frattini et al. 2003; Kornak et al. 2001; Waguespack et al. 2003). Homozygous and compound heterozygous null mutations in *CLCN7* give rise to ARO (10–15% of all ARO patients) (Frattini et al. 2003; Kornak et al. 2001). Primary neurological defects have been described in humans and mice with recessive or compound heterozygous mutations in *CLCN7*, leading to neuronal storage disease and retinal atrophy (Frattini et al. 2003; Kasper et al. 2005; Kornak et al. 2001).

Intermediate autosomal recessive osteopetrosis (IARO) is also caused by mutations in *CLCN7*. IARO is a milder form of osteopetrosis, which has, however, an autosomal recessive inheritance as in ARO (Campos-Xavier et al. 2003; Campos-Xavier et al. 2005). IARO is normally diagnosed within the first years of life, but the affected individuals are expected to reach adulthood in contrast to ARO patients (Campos-Xavier et al. 2003; Frattini et al. 2003).

Mutations in *CLCN7* also lead to autosomal dominant osteopetrosis type II (ADOII), also known as Albers-Schönberg disease, which is a heterogeneous disease in terms of severity. The penetrance has been estimated to be 66%, as several carriers do not exhibit any visual signs of disease, except for a mildly increased BMD (Benichou et al. 2001; Cleiren et al. 2001; Frattini et al. 2003; Waguespack et al. 2003; Waguespack et al. 2007). ADOII is the most common osteopetrotic disease with an estimated incidence to be as high as 5.5:100,000 (Benichou et al. 2000), although a more conservative estimate is 1:100,000 (Benichou et al. 2000).

ADOII patients are prone to delayed fracture healing (~84%) (Bollerslev et al. 1989; Benichou et al. 2000; Waguespack et al. 2007), and also have other complications, albeit with low prevalences (<20%), such as visual loss, osteomyelitis, and bone marrow failure; however, these findings appear to be secondary to the bone phenotype (Waguespack et al. 2007).

Analysis of bone biopsies from ADOII patients confirmed the increased density of the bones (Bollerslev et al. 1989). The study of the cellular phenotype of ADOII, consistently showed that high numbers of abnormally large multinuclear TRACP-positive osteoclasts were present on the inner bone surface (Bollerslev et al. 1993; Semba et al. 2000), a finding which is due to increased survival of the osteoclasts (Henriksen et al. 2006). In addition, high levels of TRACP were found in the serum of ADOII patients (Bollerslev et al. 1988; Alatalo et al. 2004; Bollerslev et al. 2000; Gram et al. 1991; Waguespack et al. 2002). The osteoclasts were devoid of ruffled border, showed high levels of intracellular vesicles and showed a dense layer of amorphous material beneath the cells that tested positive for TRACP (Bollerslev et al. 1993; Semba et al. 2000). Furthermore, increased eroded areas, but non-significantly decreased resorption rate was observed (Bollerslev et al. 1989), indicating that the osteoclasts had reduced ability to resorb bone. The mineral apposition rate (MAR), a reflection of bone formation, was increased while other osteoblast parameters remained comparable to the corresponding controls (Bollerslev et al. 1989). Consistent with the MAR findings, osteocalcin, a marker of mature osteoblasts was also found increased in ADOII patients in other studies (Alatalo et al. 2004; Del Fattore et al. 2006).

Osteoclasts from ClC-7 deficient mice and ADOII patients effectively lose their ability to induce extracellular acidification (Henriksen et al. 2004; Kornak et al. 2001), although this is still debated as some studies failed to reproduce these findings (Blair et al. 2004; Del Fattore et al. 2006).

In summary, ClC-7 deficient patients have shed further light on the complexity of remodeling, and support the notion that in acidification-attenuated patients, bone formation is regulated by the high numbers of osteoclasts rather than by bone resorption.

Osteopetrosis-associated transmembrane protein 1

OSTM1 is a putative single-pass type I membrane protein with a heavily glycosylated extracellular domain. In osteoclasts it is

expressed in lysosomes and the ruffled border of osteoclasts (Lange et al. 2006). Available evidence suggests that OSTM1 functions as a stabilizing β subunit for ClC-7 (Lange et al. 2006). Supporting the interaction is the finding that both ClC-7 and OSTM1 are under the transcriptional control of microphthalmia-associated transcription factor (MitF) (Meadows et al. 2007). Other work indicated that OSTM1 might function as an E3 ubiquitin ligase (Fischer et al. 2003), or that OSTM1 supports signaling through the wnt cascade (Feigin and Malbon 2008), and thus there are still several unanswered questions with respect to the function of OSTM1.

The phenotype of the patients with mutations in OSTM1 is very similar to that observed in other ARO patients, and thus it is severe and shows the classic osteopetrotic traits, such as very high BMD, absence of bone marrow cavities, hepatosplenomegaly and anemia (Chalhoub et al. 2003; Maranda et al. 2008; Quarello et al. 2004; Ramirez et al. 2004; Souraty et al. 2007). Studies of cells from these patients are sparse; however, a recent case report indicated that osteoclasts in vitro are morphologically normal (Maranda et al. 2008). This is in contrast to the observations made in the OSTM1 deficient grey-lethal (*gl/gl*) mice, where osteoclasts were shown to have a cytoskeletal phenotype leading to defective bone resorption (Rajapurohitam et al. 2001). This cytoskeletal trait appears to be a specific feature of *gl/gl* osteoclasts, since it is absent in the *Tcirg1* and *Clcn7* osteoclasts (Ramirez et al. 2004). OSTM1 deficient mice also show increased numbers of osteoclasts (Rajapurohitam et al. 2001), a phenomenon which was not reported in the human form (Quarello et al. 2004). Contrary to the study of *gl/gl* mice (Rajapurohitam et al. 2001) a slight decrease in osteoclast numbers was noted in this patient. In vivo, the OSTM1 deficient osteoclasts appeared elongated, instead of the classic round outline, and no resorption was observed (Quarello et al. 2004). A very recent study demonstrated an interesting phenomenon, namely that the bone phenotype of the *gl/gl* mice could not be rescued by overexpression of OSTM1 in osteoclasts using a TRACP-promoter driven approach (Pata et al. 2008). In contrast, overexpression of OSTM1 in the hematopoietic compartment using PU.1-promoter driven expression corrected the bone phenotype, as well as the B and T cell phenotypes also observed in these mice (Pata et al. 2008). These data indicate that OSTM1 is involved in intercellular cross-talk in the hematopoietic compartment; however, the complete mode of action is presently not known.

Interestingly, the *gl/gl* mice are not the only osteopetrotic mice with defects in the B and T-cells, the *oc/oc* mice, which are deficient in the a3 subunit of the V-ATPase, also have delayed maturation of these cells (Blin-Wakkach et al. 2004). Whether the B and T cells defects are similar remains to be investigated; however, these findings indicate that osteopetrosis affects the lymphoid cells through a yet to be fully understood mechanism.

The patients with OSTM1 mutations show severe neurological defects, and they actually appear to have the most severe neurological phenotype of all osteopetrosis forms (Chalhoub et al. 2003; Maranda et al. 2008). It is interesting to note that although severe primary neurological defects are present in these patients (Pangrazio et al. 2006) and similar defects are found in the ClC-7 patients (Frattini et al. 2003; Kasper et al. 2005; Kornak et al. 2001), the neuronal phenotypes are not identical (Maranda et al. 2008). Thus, there are still several issues to clarify with respect to the function of OSTM1 and the phenotype of the OSTM1 deficient patients.

Cathepsin K

Cathepsin K is a lysosomal protease mainly expressed by the osteoclasts, which is mainly active at acidic pH, and essential for the proteolytic cleavage of the type I collagen fibers that are exposed in the resorption pit following dissolution of the inorganic matrix (Bossard et al. 1996; Drake et al. 1996; Garnero et al. 1998; Gelb et al. 1996; Gowen et al. 1999).

Pycnodysostosis is a rare recessive disease, which by definition belongs to the category osteopetrosis since it is caused by an osteoclast defect, however, from a phenotypical point of view it is quite different from the other forms of osteopetrosis. The affected individuals are characterized by short stature, a large skull, retention of the teeth, and no closure of the cranial sutures (Helfrich 2003). Furthermore, a generalized sclerosis of the bones is observed, although some bones are more affected than others, as seen in the skull of these patients (Helfrich 2003). The disease causing gene encodes cathepsin K (Gelb et al. 1996), and the common denominator for the mutations is reduced enzymatic activity, leading to the bone phenotype (Donnarumma et al. 2007; Fujita et al. 2000; Haagerup et al. 2000; Ho et al. 1999; Hou et al. 1999).

The pycnodysostotic bones are characterized by defective remodeling, which leads to poor bone structure and hypomineralization, findings that are consistent with the high number of fractures occurring in these patients (Fratzl-Zelman et al. 2004; Sarnsethsiri et al. 1971; Schilling et al. 2007). Bone biopsies from pycnodysostotic patients revealed high levels of collagen fibers remaining in the resorption pit, as well as accumulations of non-digested collagen fibers in vesicles inside the osteoclasts (Everts et al. 1985).

Biochemical markers of bone turnover in pycnodysostosis demonstrated that the level of the cathepsin K generated type I collagen fragment (CTX-I) was greatly

reduced, whereas the MMP generated type I collagen fragment (ICTP) was present in high amounts, indicating compensation by these proteases (Nishi et al. 1999) In vitro cultured cathepsin K deficient osteoclasts showed complete absence of CTX-I release (Chavassieux et al. 2008). Compensation for lack of cathepsin K activity by MMPs is also seen in cultures of human osteoclasts challenged with the cysteine proteinase inhibitor E64 (Garnero et al. 2003; Henriksen et al. 2006; Sassi et al. 2000), a phenomenon also seen in cathepsin K deficient mice (Kiviranta et al. 2005). The mice have a bone phenotype that mimics the human bone phenotype to a large extent, and in vitro data from these mice parallel the data from humans (Gowen et al. 1999; Saftig et al. 1998). However, the mice have markedly increased osteoclast numbers, which has been speculated to be due to increased osteoclastogenesis, as well as decreased osteoclast apoptosis (Chen et al. 2007; Kiviranta et al. 2005).

Interestingly, there are studies showing that arg-gly-asp (RGD) sequences, which are present in type I collagen and involved in integrin binding (Fratzl-Zelman et al. 2004; Li et al. 2006), induce apoptosis in the osteoblasts, indicating that osteoblast function could be attenuated, and providing an explanation for both the poor structure of the bones formed in the pycnodysostotic patients, as well as in the cathepsin K deficient mice (Fratzl-Zelman et al. 2004; Li et al. 2006). This line is further supported by a recent case report testing the activity of parathyroid hormone (PTH) in a pycnodysostotic patient. Consistent with the distorted bone formation seen in cathepsin K deficient individuals, PTH showed no anabolic activity (Chavassieux et al. 2008), supporting the hypothesis that bone formation cannot occur in resorption pits containing high levels of collagen fibers.

Carbonic anhydrase II deficiency

CAII is an enzyme, which is highly expressed in resorbing osteoclasts (Asotra et al. 1994; Laitala and Vaananen 1994; Zheng et al. 1993). The role of the enzyme is to catalyze the conversion of H_2O and CO_2 into H_2CO_3, a process which also occurs spontaneously, albeit at a low rate. H_2CO_3 then dissociates into H^+ and HCO_3^-, and it is essential for the ability of the osteoclasts to dissolve the calcified bone matrix (Sly and Hu 1995).

Carbonic anhydrase II deficiency leads to an intermediate form of osteopetrosis in humans (Sly et al. 1983). Other effects include cerebral calcification and renal tubular acidosis (Sly et al. 1983). A wide range of mutations causing loss of CAII function underlies the phenotype (Shah et al. 2004). How the CAII deficiency affects bone turnover in general is currently not known, and the reason for the intermediate severity of the osteopetrosis phenotype is not known either, but could be related to compensation by other carbonic anhydrases, or the renal tubular acidosis also associated with CAII deficiency, since tubular acidosis is known to increase bone resorption (Sly and Hu 1995). In line with the last argument, Del Fattore et al. (2006) demonstrated the low extracellular pH could stimulate the resorptive activity of severely osteopetrotic osteoclasts through the Na^+/H^+ antiporter (Del Fattore et al. 2006).

CAII deficient mice only show a modest bone phenotype (Margolis et al. 2008), with increased numbers of osteoclasts, but reduced indices of bone formation (Margolis et al. 2008), findings which do not correlate well with the perception that osteoclasts are crucial for bone formation. However, since the mice have renal acidosis (Margolis et al. 2008; Sly and Hu 1995), and since the kidneys are highly involved in bone turnover (Favus et al. 2006; Silva et al. 2003), this phenomenon needs to be studied in further detail.

Pleckstrin homology domain containing, family M (with RUN domain) member 1 deficiency

PLEKHM1 is a recently identified protein, which is speculated to be involved in vesicular transport in osteoclasts (Van Wesenbeeck et al. 2007). A recent study showed that a mutation in the *PLEKHM1* gene led to an intermediate form of osteopetrosis, due to an intrinsic resorption defect in the osteoclasts (Van Wesenbeeck et al. 2007). The mutation caused loss of expression of the PLEKHM1 protein, which caused defective vesicular trafficking in the osteoclasts (Van Wesenbeeck et al. 2007). The PLEKHM1 deficient osteoclasts showed accumulations of TRACP activity in vesicular structures. These findings were further elaborated by a study indicating that osteoclasts with another PLEKHM1 mutation, which caused focal osteosclerosis, produced excess amounts of TRACP; however, the osteoclasts resorbed normally (Del Fattore et al. 2008b). The excessive production of TRACP was indicated to induce expression of alkaline phosphatase (ALP) activity in osteoblast cultures, indicating that TRACP plays a role in the coupling between bone resorption and bone formation (Del Fattore et al. 2008b).

The osteopetrotic *ia/ia* rat, was also shown to have a mutation leading to a truncated and non-functional PLEKHM1 protein (Van Wesenbeeck et al. 2007). The *ia/ia* rats are characterized by high numbers of non-functional osteoclasts showing absence of the ruffled border (Reinholt et al. 1999).

The *ia/ia* osteoclast phenotype is characterized by the production of abnormally high levels of TRACP (Reinholt

et al. 1999), and it will be interesting to elucidate whether the high levels of TRACP seen in these animals, as well as in the osteoclast-rich forms of osteopetroses are involved in the apparent uncoupling, or if this phenomenon is directly related to the high numbers of osteoclasts.

RANKL and RANK deficiency

RANKL, also known as TNFSF11 [tumor necrosis factor (ligand) superfamily, member 11], ODF [osteoclast differentiation factor, or OPGL (osteoprotegerin ligand)] is an essential factor for osteoclastogenesis, osteoclast function and survival (Boyle et al. 2003), and mice deficient in RANKL show osteopetrosis due to the absence of osteoclasts (Boyle et al. 2003). RANK, also known as TNFRSF11A, is the receptor for RANKL (Li et al. 2000), and it is equally essential for osteoclastogenesis, osteoclast function and survival (Li et al. 2000). A recent study showed that mutations in the *TNFSF11* gene, caused an ARO phenotype, which was characterized by absence of osteoclasts in vivo, but the ability to generate osteoclasts in vitro in the presence of RANKL (Sobacchi et al. 2007). Interestingly, the phenotype of the RANKL ARO patients develops slower than the "classical" ARO, despite the complete absence of osteoclasts (Sobacchi et al. 2007). A second recent study identified mutations in RANK, causing an ARO bone phenotype similar to that caused by the RANKL mutations (Guerrini et al. 2008). It will be very interesting to elucidate whether the difference in phenotypes of osteoclast-poor and osteoclast-rich osteopetrosis is related to signaling from osteoclasts to osteoblasts (Karsdal et al. 2007).

Other forms of osteopetrosis

Mutations in the *ITGB3* gene encoding β3 integrin in some cases lead to osteopetrosis in humans, a phenotype also seen in β3 integrin deficient mice (Horton et al. 2003; McHugh et al. 2000; Yarali et al. 2003). Furthermore, in humans, mutations in *IKBKG* gene, encoding the NFκB essential modulator (NEMO) also lead to osteopetrosis (Dupuis-Girod et al. 2002; Iotsova et al. 1997). There are some forms of human osteopetrosis without osteoclasts, for which the causing mutations have not yet been found (Balemans et al. 2005; Helfrich et al. 2007). A few of these cases showing morphologically altered osteoclasts have also been published. However, the genotypes for these have never been identified (Helfrich and Gerritsen 2001; Teti et al. 1999). However, since these cases are rare, not much is known about overall bone turnover, and they will not be discussed any further.

Animal models of osteopetrosis

There are numerous animal models of osteopetrosis and we refer to previous reviews for a detailed description (Ogbureke et al. 2005; Van Wesenbeeck and Van Hul 2005). These models can be broadly separated into models with osteoclasts and models without osteoclasts. Few human conditions homologous to the models without osteoclasts have been found, and the only known genes causing this phenotype in humans are *TNFSF11* (the RANKL gene) and TNFRSF11A (RANK) mutations (Guerrini et al. 2008; Sobacchi et al. 2007). In spite of that, several studies in mice showing osteopetrosis due to the absence of osteoclasts have been published. These are of particular interest because their bone phenotype is different from that of the models with non-resorbing osteoclasts (Karsdal and Henriksen 2007).

The remarkable finding in osteoclast-poor models is that bone formation is decreased, as seen in c-fos deficient mice (Demiralp et al. 2002). In addition, mice with no M-CSF or no M-CSF receptor have bones that are structurally disorganized indicating the loss of directionality in bone formation (Dai et al. 2004; Sakagami et al. 2005). The low bone formation phenotype appears to be present in other osteoclast deficient mice, such as the DAP12/FcR-gamma deficient mice (Koga et al. 2004; Mocsai et al. 2004; Nataf et al. 2005).

Further emphasizing the bone formation aspects of osteoclast-poor osteopetrotic mice, is the finding that PTH fails to induce bone formation in c-fos deficient mice (Demiralp et al. 2002). In contrast, in the osteoclast-rich c-src deficient mice bone formation is increased (Marzia et al. 2000), and PTH stimulates bone formation although resorption is absent (Koh et al. 2005).

The ClC-7 deficient mice and the *oc/oc* mice have recently been shown to have greatly decreased resorption, increased numbers of osteoclasts, and increased bone formation indices (Neutzsky-Wulff et al. 2008). Finally, a recent study showed that mice deficient in the d2 subunit of the V-ATPase exhibited a mild osteopetrosis characterized by increased formation and reduced resorption. Although the number of mature osteoclasts was reduced, higher numbers of pre-osteoclasts were found (Lee et al. 2006).

All in all, the osteopetrotic animal models indicate that the bone formation is regulated by the osteoclasts and that the process, to some extent, is independent of the resorptive capacity of the osteoclasts.

The coupling process revisited

The coupling between bone resorption and bone formation was first described by Frost and co-workers (Hattner et al.

1965; Takahashi et al. 1964). The coupling principle refers to the finding that bone formation in healthy adults always follows bone resorption by the osteoclasts, leading to restoration of the lost bone. Frost et al. demonstrated that bone formation in healthy adults always occurs in vacated resorption pits (Hattner et al. 1965; Takahashi et al. 1964). Later, it was shown that the number of bone forming osteoblasts correlated to the number of nuclei in the osteoclasts (Thompson et al. 1975), and, finally, Howard et al. (1981) demonstrated that resorbing bone organ cultures secreted a factor inducing bone formation.

Studies of patients with ADOII showed that these patients had normal or even increased bone formation, despite showing indications of defective osteoclast function (Bollerslev et al. 1989, 1993), a finding later speculated to be due to the increased numbers of osteoclasts (Karsdal et al. 2005). These studies have later been supported by other studies, which have shown that the osteoblast number correlates to the number of osteoclasts, not to their resorption (Alatalo et al. 2004; Del Fattore et al. 2006). Furthermore, as described in the previous section, osteopetrotic mouse models underline the importance of the presence of osteoclasts, and not necessarily their resorption, in order to maintain bone formation (Fig. 5) (Karsdal et al. 2007; Martin and Sims 2005).

These findings suggest that bone formation is ongoing despite a very low bone resorption, due to an imbalance in the normal coupling between resorption and formation. This could be attributed to anabolic "coupling" factors secreted directly by the osteoclasts, independent of bone resorption, as demonstrated in a very recent publication (Karsdal et al. 2008).

With respect to the molecular identity of the "coupling factors", traditional candidates are transforming growth factor β and the insulin-like growth factors (IGFs), which are both released during bone resorption and produced by the osteoclasts (Baylink et al. 1993; Hayden et al. 1995). Furthermore, bone morphogenetic proteins (BMPs) and platelet derived growth factors (PDGFs) are released during resorption (Mundy et al. 1999). Independent of bone resorption, forward–reverse signaling through EphrinB2, expressed by osteoclasts, and EphB4, expressed by osteoblasts, was shown to augment bone formation, while reducing osteoclastogenesis (Zhao et al. 2006). Furthermore, Del Fattore et al. (2008b) showed that increased secretion of TRACP by osteoclasts with a mutation in PLEKHM1, could induce bone formation in vitro, and thereby indicated that TRACP could function as an osteoclast-derived "coupling factor". Finally, a very recent study showed that osteoclast-mediated production of Cardiotrophin-1 could augment bone formation by osteoblasts under some circumstances (Walker et al. 2008).

Thus, there are several candidate molecules for the "coupling factor", both molecules derived from bone resorption and directly from the osteoclasts, and further understanding of this mechanism will aid the development of novel generations of anti-resorptive treatments for osteoporosis.

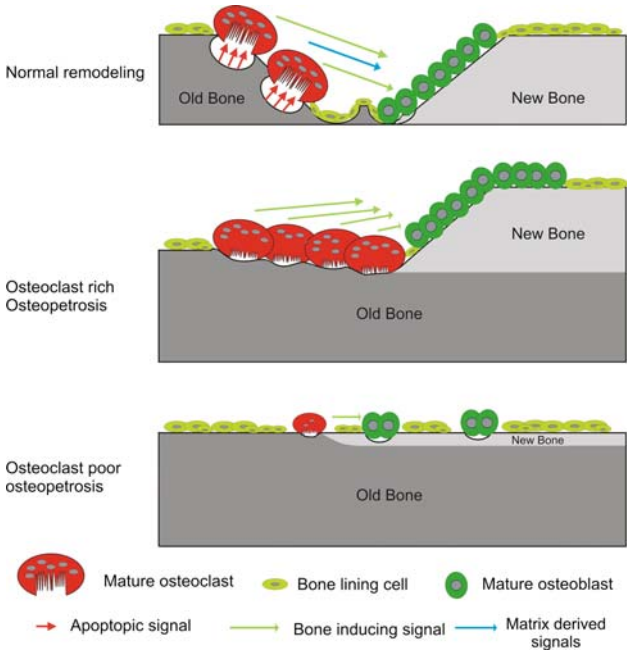

Fig. 5 The coupling of bone formation to bone resorption. Under normal circumstances bone formation follows bone resorption leading to restoration of the removed bone matrix. In some osteopetrotic cases, the osteoclast number is increased, whereas resorption is dimished as illustrated by the remaining old bone (*dark gray*). In contrast bone formation (*light gray*) appears to follow the number of osteoclasts, indicating the resorption can be attenuated without effects on formation. In osteoclast-poor forms of osteopetrosis, little resorption takes place again illustrated by the remaining old bone (*dark gray*). However, since no osteoclasts are present bone formation (*light gray*) is also distorted, and thus the phenotypes are less severe than the osteoclast-rich ARO phenotypes

Future perspectives

The design of novel treatments for osteoporosis has long been inspired by osteopetrosis. This rare condition provides unique insight into the effects of selectively removing specific components of the osteoclast function. As loss of those components invariably results in increases in skeletal mass, they all qualify *a* priori as potential targets for therapeutic intervention in osteoporosis. However, a general problem with existing antiresorptive treatments for osteoporosis is an associated decrease in bone formation resulting from the coupling between resorption and formation (Hansdottir et al. 2004; McClung et al. 2006; Ravn et al. 1999a, b). Even combined treatment with bisphosphonate and PTH has proved ineffective in two key independent studies (Black et al. 2003; Finkelstein et al. 2003). Instead, studies using novel antiresorptives inspired by osteopetrotic phenotypes

indicate that bone resorption and bone formation can be effectively dissociated (Hannon et al. 2005; Karsdal et al. 2005; Rzeszutek et al. 2003; Schaller et al. 2004; Visentin et al. 2000). The common denominator of those agents is their ability to inhibit resorption without decreasing osteoclast numbers, and a likely explanation for their action is that continued bone formation may proceed unimpaired when and if an operative signaling system enables communication from osteoclasts to osteoblasts, as seen in specific cases of osteopetrosis (Karsdal et al. 2007).

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