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HUMAN OSTEOPETROSES AND THE OSTEOCLAST V-H$^+$-ATPASE ACIDIFICATION SYSTEM

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1. ABSTRACT

Osteopetroses are a heterogeneous group of human genetic diseases characterized by generalized increase in bone density due either to a lack of osteoclast population or defect in osteoclast function. Current knowledge of the pathogenesis suggests defects that may be either intrinsic to osteoclast-monocyte lineage or extrinsic to the mesenchymal cells that support osteoblast ontogeny and activation. Four clinically distinct forms of human osteopetroses currently recognized are the infantile malignant autosomal recessive form, the intermediate autosomal recessive form, the adult benign autosomal dominant osteopetrosis type I, and the autosomal dominant osteopetrosis type II. Propensity to fracture is high in all types of osteopetrosis, and other characteristic clinical problems include hematologic and metabolic abnormalities, infections of affected bone, and neurologic sequela. Among the infantile malignant clinical forms 50-60% of patients present with defects in the OC116-KDa (also refers to ATP6i, TCIRGI, a3) subunit of the osteoclast vacuolar H+-ATPase (V-H+-ATPase) proton pump. Approaches that have been applied to the treatment of osteopetrosis include those aimed at stimulating host osteoclasts. These approaches however have met with little success, and it would appear that the future for the successful treatment of osteopetrosis lies with bone marrow transplantation. Various animal models mimicking some of the clinical subtypes of osteopetrosis have been generated in efforts to elicit further understanding of the pathogenesis. This review is an update on the various phenotypic presentations of human osteopetroses alongside their known animal models. Further studies on these animal models will not only expand our basic understanding of the molecular mechanisms of osteopetroses, but will also aid our ability to develop therapeutic means of intervention in diseases involving osteopetroses.

2. INTRODUCTION

Skeletal homeostasis, including normal bone density, is determined by a balance between matrix formation and mineralization functions of osteoblasts, and the resorption of mineralized bone by osteoclasts. It is now well established that osteoclasts originate from pluripotent hematopoietic stem cells, from which other leucocytes are derived, by the fusion of mononuclear cell precursors to form multinucleated giant cells specializing in lacuna resorption (1-3). Walker first advanced evidence supporting this conclusion following successive experiments with parabiosis marrow transplant (4;5). The osteoclast lineage shares marrow precursors with monocytes and macrophages, including committed precursor (CFU-GM) for cells of this lineage (6-8). Recent studies involving transgenic animal and stem cell differentiation procedures have provided additional unequivocal evidence of the hematopoietic nature of osteoclast, while at the same time showing that the chondrocytes and osteoblasts originate from mesenchymal stem cells (9;10).

Osteoblasts, chondrocytes and their mineralized matrices, together with stromal and endothelial cells, provide the microenvironment for homing of osteoclast precursor cells. Osteoblasts/stromal cells produce cytokines including macrophage-colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) that induce and modulate growth and differentiation of the precursors to mature osteoclasts. M-CSF binds to its receptor, c-Fms, present on osteoclast precursors, providing the signals for macrophage survival and proliferation (11). Binding of RANKL to its receptor RANK results in the recruitment of TRAF family proteins such as TRAF6 which is essential for osteoclast cytoskeletal organization and resorbing activity (12). To date, at least five distinct signaling cascades mediated by protein kinases are induced during osteoclastogenesis and activation — inhibitor of NF- κB kinase (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK), and Src pathways (13) and most of these signaling cascades function downstream of TRAFs to activate osteoclast specific genes. NFAT2, identified recently, also plays an integral role in the RANKL-induced transcriptional program during terminal differentiation of osteoclasts (14). RANKL evokes $[Ca^{2+}]i$ oscillations that lead to calcineurin-mediated activation of NFAT2 and therefore triggers a sustained NFAT2-dependent transcriptional program have been reported in this pathway (14). Multiple overlapping signal transduction pathways of physiological significance in osteoclast differentiation are yet to be fully elucidated.

A common cellular defect to all forms of human osteopetrosis is impaired osteoclast bone resorption. Bone resorption, a function of osteoclasts, forms an essential half of the coupled process of bone remodeling required to maintain normal skeletal homeostasis. The other half involving bone matrix synthesis is modulated by the osteoblasts. Activation of osteoclasts starts with their adhesion to bone matrix (15). Thereafter, cytoskeletal reorganization, cell polarization, and formation of unique membrane areas for bone resorption take place (15;16). During resorption, osteoclast microfilaments form a specific ring-like "sealing zone" that mediates tight attachment of the cell to mineralized bone matrix (15;17). The sealing zone surrounds the ruffled border, a convoluted membrane area formed as a result of insertion of vesicles and active directional secretion (17;18). Bone resorption requires osteoclast secretion of protons and protease into the interface compartment between the cell and the bone matrix (19;20). Protons are extruded by a pump belonging to the class of vacuolar-type H^+ ATPase (V-H^+ ATPase) that is present in high concentration on the ruffled border (apical surface) of the resorbing osteoclast so as to produce a low pH in the microenvironment (15;19;20). This allows for the dissolution of bone mineral and the digestion of organic matrix of bone (21) (Figure 1).

3. CLINICAL PERSPECTIVE AND GENETIC ABBERATIONS OF HUMAN OSTEOPETROSES

Osteopetroses is one among several disorders causing osteosclerosis of the trabecular bone and/or hyperostosis of the cortical bone (22). Although osteosclerosis is widespread, osseous changes occur most severely at the base of the skull resulting in unilateral or bilateral obliteration of cranial nerve foramina (22-24). These changes lead to serious clinical symptoms such as deafness, facial paralysis, and optic nerve compression resulting from their corresponding cranial nerve palsies (23). Although rare, both human osteopetrosis and osteosclerosis are quite common among naturally occurring and genetically engineered mouse mutants (24;25). Over 20 different types of OP have been described in both animals and humans (26-32) with at least four clinically different types defined in humans (33;34). They include the infantile malignant autosomal recessive OP (arOP), intermediate variants of arOP, and two forms of the adult onset types inherited in autosomal dominant fashion and referred to as autosomal dominant OP types 1 and 2. Furthermore, patients with atypical symptoms continue to be identified, suggesting that there are additional forms (22).

3.1. Human infantile malignant autosomal recessive OP (arOP)

Human infantile malignant autosomal recessive osteopetrosis (arOP) is a rare genetic disorder in which defective osteoclasts fail to resorb bone resulting in excessive accumulation of mineralized bone and abnormal hematopoiesis (22;35). The abnormal hematopoeisis (extramedullary) is a consequence of inadequate marrow spaces (7;36). arOP inherited as an autosomal recessive disorder, is the most clinically severe type of OP and is usually fatal within the first few years of life (often before school age) in the absence of bone marrow transplantation (22;37;38). Patients with arOP usually die from complications of anemia and infection (33;38). Affected infants typically present in the first few months of life with failure to thrive, severe hepatosplenomegaly, pancytopenia, macrocephaly, and visual and auditory impairments due to cranial nerve compression (22;37;38). The exact incidence of arOP is unknown but is estimated to be about 1:200,000 or 5:1,000,000 live births with no known gender or ethnic

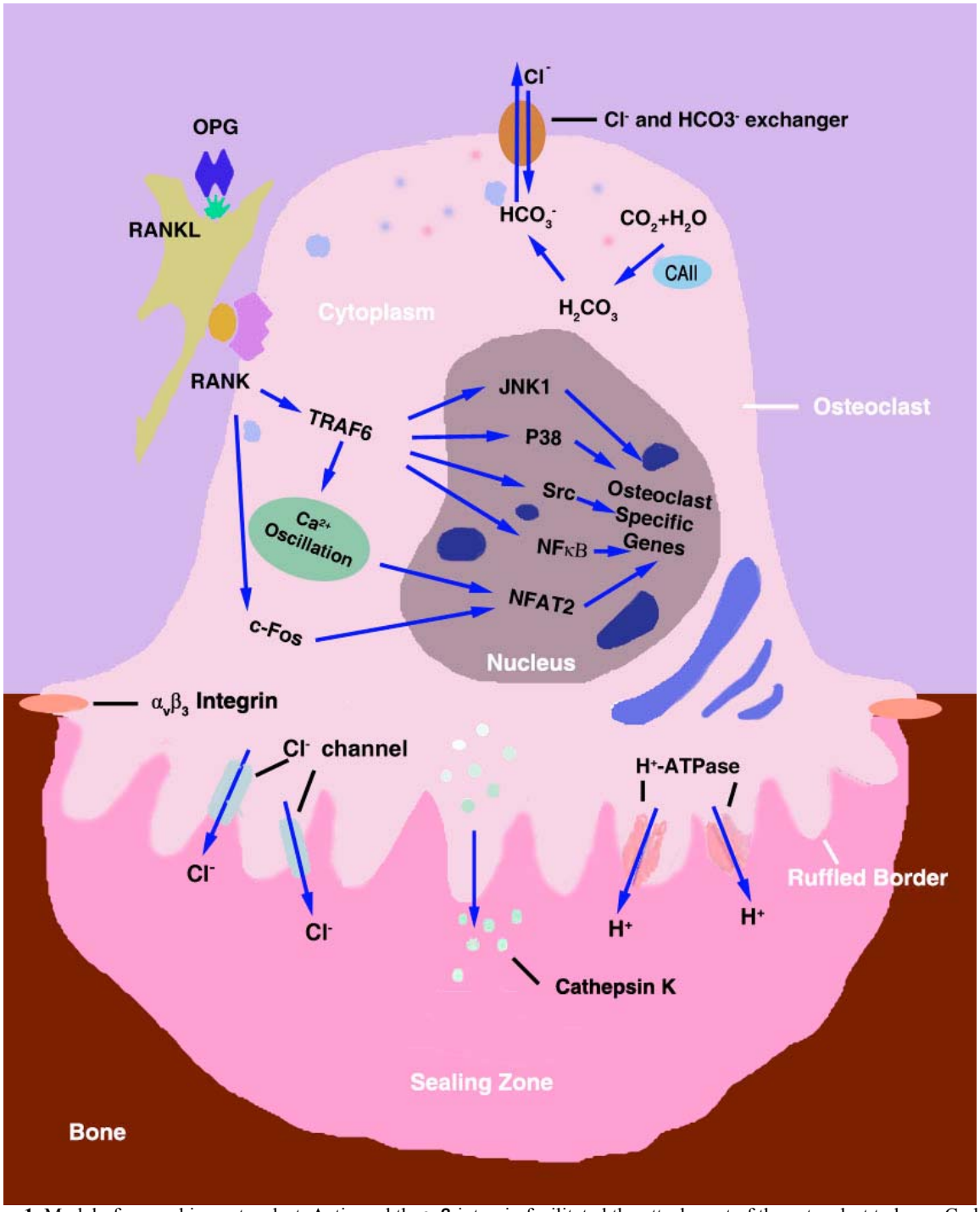

Figure 1. Model of a resorbing osteoclast. Actin and the$\alpha_v\beta_3$integrin facilitated the attachment of the osteoclast to bone. Carbonic anhydrase II (CAII) generates proton and bicarbonate from carbon dioxide and water to achieve acidification of the resorption lacunae and begin the process of bone demineralization. Proton is then actively transported through the action of ATP6i across the membrane of the ruffled border. Chloride channels balance the charge of ions across the membrane through coupling with the proton pump. Finally, excess bicarbonate is removed through the basolateral membrane by passive exchange with chloride. RANKL, the critical gene of osteoclast differentiation, activation and survival of mature osteoclasts, binds to RANK and activates at least five distinct signaling cascades: inhibitor of NF-kB kinase (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK), and Src pathways NFAT2 is an recently identified molecular that plays an integral role in the RANKL-induced transcriptional program during terminal differentiation of osteoclasts. RANKL evokes $[Ca^{2+}]i$ oscillations that lead to calcineurin-mediated activation of NFAT2 during osteoclast differentiation.

predilection (33;38). There is a mortality rate of about 70% for the first six years of life, which spirals to nearly 100% by the end of the first decade of life (38;39). At the moment, the only hope of a curative therapy for arOP appears to be stem cell transplantation (36).

Two different anomalies of osteoclasts have the potential to lead to inadequate bone resorption resulting in increased bone mass (31;40;41). There is either a defect in the differentiation of osteoclast progenitors precluding the formation of osteoclasts, or if osteoclast formation does occur, a failure to activate differentiated mature osteoclasts (41-43). Most forms of human arOP however display increased number of well differentiated osteoclasts with functional defect (41-43). In patients with arOP where it appears that the number of osteoclasts is normal these osteoclasts lacked ruffled borders (28;39). Three major gene mutations have been characterized to date as being responsible for human arOP (28;29;32;44;45). The various clinical manifestations of arOP patients are thought to reflect the genotype heterogeneity

A majority (58%) however involve mutation in the gene encoding the OC116-Kda subunit of the V-H^+ ATPase pump (28;44;45) that is unique and necessary for osteoclast-mediated extracellular acidification. This gene is referred to variously as *ATP6i*, *OC116* or *TCIRG1* (28;46). Heaney *et al.* (47) described two bedouin kindred affected with arOP as having significant linkage to the 11q12-13 region of the OC-116 gene. In a study of nine children with infantile malignant osteopetrosis by Frattini *et al.* (28), five had a null mutation in the TCIRGI gene encoding OC-116, and three shared the same splice acceptor site mutation. These mutations caused abnormal transcription with no protein synthesis. Another study by Kornak *et al.* (48) revealed a mutation in the TCIRGI gene in five out of 10 children with infantile malignant osteopetrosis. Following these studies, Michigami *et al.* (49) reported two novel heterozygous mutations in the OC116-KDa subunit of the V-H^+-ATPase gene in a patient with infantile malignant osteopetrosis. The first was a mutation at the second position of the splice donor site of intron 19, while the second involved a deletion/insertion mutation in exon 9. The dinucleotide at the splice donor site of intron 19 was mutated from GT to GC in apparent violation of introns' GT-AG rule (49). This violation resulting from abnormal slicing was confirmed by RT-PCR to amplify the TIRC7 transcript using total RNA from the patient's liver specimen (49). The deletion/insertion mutation in exon 9 resulted in a frame shift and a premature stop codon, suggesting that transcripts derived from this allele cannot produce normal protein (49).

A second well-characterized genetic abnormality for osteopetrosis in humans involves defects in the chloride channel homolog, CLCN7. Studies evaluating the abnormalities in the chloride channel coupled to osteoclast H^+-ATPase as a cause of osteopetrosis indicate that these defects have a less straightforward effect on osteclastic activity. Nevertheless, the mechanisms of CLCN7 dysfunction is better understood than those of other members of the chloride channel family. Overall, defects in the CLCN7 gene appear less frequently as a cause of osteopetrosis than mutations in the TCIRG1 gene (50). CLCN7 is one of the ClC-family of voltage-dependent chloride channels with very high expression within the ruffled border of osteoclasts (29). CLCN7 encodes a late endosomal/lysosomal chloride channel CLC-7 involved in osteoclast hydrochloric acid (HCl) secretion. The CLC-7 channel is responsible for dissipation of the positive potential in the resorption lacuna, which acts as an "extracellular lysosome" and is acidified by a V-H^+ pump.

Kornak *et al.* (29) identified CLCN7 mutations in a patient with infantile malignant osteopetrosis. In the series recently reported by Frattini *et al.* (44), about 15% of patients with severe arOP result from CLCN7 mutations. Mutations in the CLCN7 are more heterogeneous with some being null mutations and others missense mutations (44). Cases resulting from null mutations cause severe arOP while those of missense mutations maintain residual functions or even gain-of-function, behaving as dominant mutations. This is consistent with clinical manifestations, which range from extremely severe to clinically asymptomatic manifestations. Clinically, severe osteopetrosis resulting from mutations in CLCN7 are similar to those resulting from TCIRG1 mutations, including presentation in infancy, and possible neurologic changes (29;44). In the CLCN7 mutation retinal degeneration, independent of optic nerve compression is likely to represent a specific stigma of the disease (29;39). Some mutations of the CLCN7 genes in humans result, instead, either in the so-called type II benign autosomal dominant osteopetrosis (a milder syndrome known as Albers-Schonberg disease discussed below) or in intermediate forms of osteopetrosis (44;51).

The spontaneous mouse model that most closely resembles the severe human arOP is the grey lethal (*gl*) mutant (52). As in humans, functional rescue of the *gl/gl* phenotype can be obtained by bone marrow transplantation, and this provides evidence of a cell-autonomous defect (5;52). Recently, Quarello *et al.* (53) reported the first case of human severe arOP resulting from a mutation in the grey-lethal (GL) gene in a 9-day-old male. This was a homozygous point mutation causing a skipping of exon 5. The patient presented with substantial hepatomegally, cytopenia, and progressive liver failure, and died on the 31st day of life due to multiorgan failure. Skeletal radiographic changes in this patient were noted as early as the second week of life, and presented as a generalized increase in bone density with loss of cortico-medullary differentiation. Histopathologic evaluation showed typical osteopetrotic changes with absence of resorptive activity, lack of Howship's lacunae, reduced marrow spaces, slightly decreased number of osteoclasts, and evidence of osteoclast morphological alterations (53).

3.2. Intermediate forms of arOP

A subset of patients with intermediate forms of autosomal recessive OP has been characterized in recent years (54). These patients exhibit mild symptoms often compatible with long-term survival. These less severe genotypes of arOP are more commonly studied because of

the long survival and better conditions of the patients. The first of these defects described in human was an autosomal recessive condition with lack of carbonic anhydase II (CAII) isoenzyme activity (32). The CAII gene controls the production of carbonic acid and protons. The clinical presentations have been variable: patients have presented with bones that were initially very dense but with marrow spaces developing later in life (32). Yet there have been severe cases presenting with nerve compression syndromes, growth retardation, psychomotor delay, and mental retardation (32;55). Concurrent with osteosclerosis, patients present with renal tubular acidosis (32;50) because the carbonic anhydrase type II is strongly expressed by renal tubular cells. Patients however often do not present with hematological failures (32;50). Radiographically, there is diffuse increase in bone density, with metaphyseal widening and evidence of intracranial calcification. The cerebral calcification present in these patients accounts for the synonym "marble bone disease" for the carbonic anhydrase II isoenzyme deficiency-associated arOP.

Approximately 5% of patients with CAII deficiency-associated arOP are among about 50 cases of mildly recessive arOP reported worldwide (50). The molecular defects in *CAII* gene appear relatively specific to particular geographic regions. These are patients of Arab descent (55;56), and Caribbean Hispanic patients (57). CAII deficiency arOP responds to bone marrow transplantation therapy, which corrects the hematological and bony manifestations, and arrests the formation of additional cerebral calcification (58). The renal tubular acidosis however remains unchanged after transplantation.

3.3. Autosomal dominant OP type I (ADOI)

The autosomal dominant osteopetroses (ADOP) are benign forms of osteopetroses that are quite compatible with survival into adult life because of their mild phenotypic presentation. These conditions are therefore often detected fortuitously on radiographs. Only a minority of ADOP patients exhibit mild anemia, and most patients are asymptomatic. Initially based mainly on radiographs and a few clinical differences, two subgroups of ADOP, ADOI and ADOII, was recognized (59). It is now known that the heterogeneity accounting for ADOI and ADOII is explained by mutations in different genes (26;60;61).

ADOI is extremely rare with only cases from three families reported at the time of this update (33). Through a linkage study on two extended families, Van Hul *et al.* (60) demonstrated that the gene involved in ADOI is located in the chromosome 11q12-13 region. This region corresponded to that previously identified as the locus of a gene causing the "high bone mass" (HBM) phenotype (60;62). The HBM phenotype is notable for a very high bone density without any clinical implications (62). Identification and subsequent characterization of this gene(s) will reveal whether ADOI and HBM are allelic and caused by different mutations in the same gene or whether two genes from the same chromosomal region are involved in these two conditions (63). Clinically, a small number of ADOI patients have symptoms of bone pain but without evidence of increased fracture seen in other types of osteopetrosis (64). Radiologically, ADOI patients show generalized diffuse osteosclerosis notably of the skull, spine, and long bones (63). Histology shows persistence of calicified cartilage in early life, which improves with age. Lamellar bone is normal and no abnormality is present in the cortical remodeling.

3.4. Autosomal dominant OP type II (ADOII)

ADOII, sometimes called Albers-Schonberg disease, is the most common form of osteopetrosis (26). Many, if not all, cases of ADOII are caused by a mutation in the CLCN7 chloride channel (26;44;51;65). Here, the mutant CLCN7 gene is thought to act in a dominant-negative manner, as opposed to the malignant form in which the loss-of-function mutation does not affect heterozygous individuals (51). Clinically, patients with ADOII present with frequent fractures, possible cranial nerve palsies, and osteomyelitis (50). More severe phenotypes approximating those of recessive disease have also been reported (50). Radiological presentation is typified by the presence of the so-called "bone-within-bone" (endobones), the "Rugger-Jersey spine" resulting from thickening of the vertebral endplates, and the selective sclerosis of the skull base (64). Like in malignant osteopetrosis, abnormal lining of bone matrix by mineralized organic matrix without recognizable collagen fibers has been reported in patients with ADOII (66). However, lamellar bone is normal and no abnormality is seen in cortical remodeling.

Early linkage analysis studies assigned the gene that causes ADOII to chromosome 1p21 (67). However, subsequent linkage studies apparently reversed this earlier assignment by reassigning ADOII to a gene on chromosome 16p13.3 (68-70). The apparent conflict in assignment is because of coincidental cosegregation in the earlier case (67). Therefore ADOII might actually be genetically homogenous, being caused by a gene on chromosome 16p13.3.

3.5. Reported conditions associated with human osteopetroses

3.5.1. Human Pycnodysostosis

Besides the clinical and genetic parameters that enable the diagnosis of osteopetrosis to be made, there are several reports of specific clinical entities occurring side by side with osteopetrosis in frequencies or fashions that enable an association between osteopetrosis and the entity to be made. Human pycnodysostosis is caused by the mutations in cathepsin K gene with the phenotype of a general osteosclerosis leading to short stature, a disproportionately enlarged skull with non-closure of anterior fontanel and cranial sutures, loss of the mandibular angle and dental malocclusion with retention of deciduous teeth, hypoplastic clavicles, and short and clubbed fingers with hypoplastic or absent terminal phalanges (71).

3.5.2. Glanzmann's thrombasthenia

Yarali *et al.* (34) reported the case of a five-year-old child whose malignant arOP was "complicated" by *Glanzmann's thrombasthenia* (GT) at the time of presentations. Glanzmann's thrombasthenia is a rare

hereditary qualitative platelet disorder characterized by a lifelong bleeding tendency due to qualitative and quantitative abnormalities of the platelet integrin $\alpha_{IIb}\beta 3$. Several mutations in either the α_{IIb} (glycoprotein (GP) IIb) or the β3 (GP IIIa) subunits have been reported in GT. β3, which complexes with the αv subunit to form the αvβ3 (vitronectin) receptors, is present on both platelets and osteoclast, among other cell types (53), and αvβ3 has been specifically implicated in osteoclastic bone resorption. McHugh *et al.* (72) demonstrated the development of histologically and radiologically evident osteosclerosis in β3 integrin knockout mice. Recently, Feng *et al.* (73) reported the generation of mice with six point mutations known to mediate β3 integrin signaling. Of the six point mutations, only S752P substitutions failed to rescue the spreading and resorptive capacity of osteoclasts. Interestingly the same S752P substitution mutation is present in variants of GT (73). These constellation of observations led Yarali *et al.* (34) to speculate that their patient might have a mutation in β3 integrin that affects both the platelets and the osteoclasts. It is noteworthy however that both GT and malignant arOP occurs more frequently in region where consanguineous marriages are common. This adds to the speculation as to whether the joint occurrence of GT and arOP is mere coincidence, or whether there exist a common causal molecular pathway. The resolution of this question would probably await further genetic analysis of future cases.

3.5.3 Severe pulmonary hypertension

Severe pulmonary hypertension is a frequent complication of stem cell transplantation for malignant arOP. Stewart *et al.* (74) reported the development of acute pulmonary hypertension (PAH) in eight out of 28 patients (29%) treated for malignant arOP by allogenic stem cell transplantation. Among the symptom was evidence of veno-occlusive disease (VOD) in three of the eight patients with PAH. The authors concluded that the absence of VOD in the other five with PAH suggested a separate disease mechanism for PAH. While it is possible that PAH in these patients might be accounted for by a separate disease mechanism, the deduction of the authors based on the development of VOD by some and not the others does not, standing alone, support this opinion. It may well be that the development of PAH and then VOD in malignant arOP constitutes a continuum of complications associated with the arOP.

3.5.4. Lymphoedema-Anhydrotic Ectodermal Dysplasia-Immunodeficiency Syndrom (OL-EDA-ID "syndrome")

Recently, Dupuis-Girod *et al.* (75) reported two cases of X-linked osteopetrosis occurring alongside lymphoedema, anhidroitic ectodermal dysplasia, immunodeficiency, and incontinentia pigmenti (OL-EDA-ID). The two male children were unrelated. These patients had mutations in NF-κB essential modulator (NEMO), which encodes an essential component of the NF-κB signaling pathway (75), and were hemizygous for the same NEMO stop codon hypomorphic mutation. Both patients exhibited the classical clinical features incorporated by the components of OL-EDA-ID (75). Worthy of note however is the fact that X-linked dominant incontinentia pigmenti (IP) has been found to be caused by loss-of-function mutations in NEMO. Because of the occurrence of identical clinical features with the same phenotype in these two unrelated patients, the authors concluded that OL-EDA-ID is a genuine syndrome (75).

3.5.5. "Acquired" (Iatrogenic) osteopetrosis

Treatment with Biphosphonate in children with genetic skeletal diseases such as osteogenesis imperfecta, juvenile osteoporosis, and fibrous dysplasia is common. Recently Whyte *et al.* (76) reported a case of "acquired osteopetrosis" resulting from a longterm treatment with biphosphonate in a 12-year-old white boy. We suggest that "*iatrogenic osteopetrosis*" is a more appropriate term, and should replace "acquired osteopetrosis", because the condition is an unintended outcome of the effects of drugs therapeutically intended for a pre-existing condition. Evidently, the so-called acquired osteopetrosis is a consequence of abnormal bone modeling and increased bone density possibly due to inhibition in the recruitment and function of hitherto functionally normal osteoclasts. The inhibition is cumulative presumably over extended biphosphonate therapy.

4. OSTEOCLAST V-H^+ATPase ENZYME SYSTEM AND ANIMAL MODELS OF HUMAN OSTEOPETROSIS

4.1. Osteoclast V-H^+ ATPase enzyme system

The V-H^+-ATPases are enzymes first identified as electrogenic proton pumps of the vacuolar system in lysosomes and chromaffin granules (77). They are found in diverse eukaryotic endomembrane compartments and plasma membranes of epithelia, macrophages, and specialized polarized cells (78-80). Among the macrophage polykaryons (giant cells), the osteoclasts are the only ones with massive expression of cell-surface V-H^+ ATPase required for the high energy acid secretion that dissolves bone minerals (78). However, the V-H^+ ATPases have not been localized in the nucleus and mitochondrion (81). Their functional role reflects this broad distribution, ranging from processing receptor-ligand complexes in endosomes to osteoclast-mediated dissolution (81). Among many others, issues yet to be resolved include the mechanisms by which V-H^+-ATPases are targeted to their cellular sites and how differential regulation of the enzyme is achieved at the different locales.

The V-H^+-ATPase system is a multi-subunit enzyme with two functional domains, V_1 and V_o. V_1 comprises a complex of peripheral subunits that attaches to Vo, which is a complex of integral proteins (21;79;80;82). The V_1 (catalytic) domain consists of subunits A through H with three catalytic sites at the interface between the A and B subunits, similar to ATP synthase (21). On the other hand, the V_o (membrane-spanning) domain, which forms a proton pathway, consists of six copies of a 15KDa proteolipid and associated proteins of 110 to 116Kd (21).

The osteoclast-specific C116-KDa subunit of the V-ATPase is a 116-kd glycoprotein encoded by the *ATP6i*

gene, also known as *TCIRGI* (83;84). Because mutations in the *ATP6i* gene are responsible for the most severe type of arOP, it is pertinent to explain the evolution of certain terminology and "synonyms" associated with the subunits of the V-H^+ ATPase pump. *Atp6i* refers to the a3 isoform of the mouse homologue of human *OC-116* characterized by Li *et al.* ([83]; *Atp6i,* for V-proton pump H^+ transporting member I). Mouse *Atp6i* includes approximately 4kb of the putative promoter region and 20 exons (83). The sequence derived from these exons is highly conserved with the human *OC116 cDNA*. Northern analysis of various mouse tissues showed *ATP6i* predominantly and selectively expressed in osteoclasts, a finding consistent with a previous observation with the human *OC116* (46;82). Alignment of the mouse *Atp6i* gene exon sequences to the human *OC-116* cDNA revealed that there is a strong homology at both the nucleotide (82%) and the amino acid (80%) levels (83). In contrast, the sequence homology with the human gene at the 5' and 3' untranslated regions is only 60% and 56%, respectively (82;83;85). The evolution of the TICR7 synonym for OC-116/ATP6i originated with the 1998 report of Utku *et al.* (86) in which the authors identified TICR7 as a novel T cell membrane protein that plays a central role in T cell activation and subsequent immune response related to enhanced allografts responses. Comparative sequence analysis revealed that TICR7 possessed considerable sequence homology to human *OC-116* (86;87). Furthermore, both were alternate spliced products of the same gene located on chromosome 11q13.4-q13.5 (86;87). However, the TIRC7-specific transcript, but not the OC-116, is present in alloactivated human T-cells (87). Nevertheless, the structural relationship between TICR7 and OC116 suggests that there is similarity between the function of both proteins, and explains the interchangeability of the terms *OC-116*, *ATP6i*, and *TICR7* in reference to the gene encoding the a3 subunit of the V-H^+ ATPase pump.

The other two isoforms (a1 and a2) of the V-H^+ ATPase *a*-subunit have been identified together along with the a3 only in mouse (82;85). Separate genes encode each of these isoforms with their products expressed differentially in various mouse tissues (82). The a1 isoform was highly expressed in brain and osteoclasts, and exhibited high identity with a-subunit from clathrin-coated vesicles of rats and bovine brain (82;88;89). The a2 subunit showed 91% identity to the subunit purified from bovine lungs, although no a2 expression was detectable in mouse lung on Northern blot (79;82). In their reconstruction experiment, Peng *et al.* (89) demonstrated that a2 is associated with functional V_o, indicating that it is a genuine isoform of the 116KDa subunit of V pumps.

Toyomura *et al.* (82) reported the presence of three transcripts for the a2 and two transcripts for a3 resulting from a possible alternate splicing and accounting, to some extent, for variation in the a-subunits. This variety of isoform combinations is enough to create the diversity of V-H^+-ATPases, accounting for different pH in acidic compartments (82). The different subunits also may contribute to the subcellular localization of the entire enzyme (82). The a1 and a2 isoforms contain one putative N-linked glycosylation site, while a3 has three sites (82). In osteoclasts, the B2 isoform is specifically expressed in the plasma membrane, and induced in osteoclasts generated from human blood monocytes (82;90). The a1, a2, and a3 isoforms are synthesized in osteoclasts, but only a3 is induced specifically and localized in the plasma membrane (82). This suggests that the V-H^+-ATPase subtype having B2 and a3 is an inducible complex involved in osteoclast-mediated bone resorption (82). It thus appears that subunit isoforms of the V-H^+-ATPase are alternately regulated, accounting for the differences in V-H^+-ATPase activity in different tissues. Furthermore, a comparison of the amino acid sequence of *ATP6i* (a3), originally cloned by Mattsson *et al.* (91) with a1 showed a 47% amino acid homology, strongly suggesting that this isoform of the OC-116 is osteoclast-specific.

In osteoclast, carbonic anhydrase II (CAII) catalyzes carbon dioxide (CO_2) and water to form carbonic acid (H_2CO_3), which subsequently dissociates into bicarbonate (HCO_3^-) and proton (H^+). Osteoclasts use the V-H^+-ATPase as a key component of the regulated pathway for bone resorption by the translocation of large numbers of V-H^+-ATPase from cytoplasmic stores to the ruffled membrane (17;21;78). Chloride-channel charge coupled to the V-H^+ ATPase preserve the electroneutrality of the ruffled membrane. The low pH of the resorptive space, coupled with osteoclast secretion of lysosomal hydrolases, result in degradation of bone matrix and dissolution of mineral (21).

A cDNA encoding a novel human cysteine protease was cloned independently by several groups including ours (92-95), and was variously named cathepsin X, cathepsin O, cathepsin O2., and cathepsin K. All four sequences are identical and represent the human equivalent of the OC2 gene cloned from rabbit osteoclasts (OCs) (96). High level expression of this enzyme has been detected in OCs, but not in other human cell lines and tissues (97;98). In contrast, mRNA for cathepsin L, B and S were either absent or expressed at very low levels in OCs (98). These data demonstrate that cathepsin K is abundant only in OCs, and that this enzyme represents greater than 98% of all cysteine proteases in this cell type. Bromme et. al. demonstrated that cathepsin K is a highly active cysteine protease that is capable of hydrolyzing extracellular matrix proteins such as collagen and elastin (99). Type I collagen, the major structural protein component in bone, is efficiently hydrolyzed at pH values above 5.5 by cathepsin K, a pH where only low, or no activity is observed for cathepsin L and cathepsin B.

Furthermore, alphavbeta3 ($\alpha v\beta 3$) integrin is pivotal to the resorptive process through its role in linking the adhesion of osteoclasts to the bone matrix with the cytoskeletal organization, and the polarization and activation of these cells for bone resorption (100). Integrins are transmembrane proteins that comprise ✿ and ß subunits. These heterodimers mediate cell-cell and cell-matrix interactions and generate intracellular signals when occupied by ligands (101), or upon treatment of cells with growth factors or cytokines (102). Osteoclasts highly express the

Table 1. Human Osteopetrosis Disease Genes and their mouse models Associated With Impaired Osteoclast Function

Mutated Gene	Phenotype in Human Cases	Defects in Osteoclasts	Association with Acid Secretion and role	Reference Human Disease	Reference Mouse Model
Atp6i	arOP	Inability of extracellular acidification	Yes; a pump mediate osteoclast extracellular acidification	28,48,49	46, 104
CLCN7	arOP & ADOII	Inability of extracellular acidification	Yes; a channel coupled to H^+-ATPase mediate osteoclast extracellular acidification	26, 44, 51, 65	29
CAII	Intermediate forms of arOP arOP	Inability of carbonic acid and protons generation	Yes; an enzyme controls the production of carbonic acid and protons;	32, 55-57	-
Cathepsin K	Pycnodysostosis	Inablity of collagen degradation	No; a cysteine protease capable of hydrolyzing extracellular matrix proteins functions in low PH environment	71	107, 108, 115
alphavbeta3 integrin	None found	Inability of resorbing bone matrix	Yes; a critical protein in osteoclast migration and formation of the sealing zone	-	72
Csf1	None found	Devoid of osteoclasts	No; a critical cytokine in the differentiation of osteoclast precursors	-	116
PU.1	None found	Devoid of osteoclasts and macrophages	No; a transcription factor associated with the M-CSF pathway;	-	10
RANK	FEO, ESH, and early onset Paget's disease	Devoid of osteoclasts	No□a critical cytokine in the differentiation and activation of osteoclast	-	117
NF-kB 1 and NF-kB 2	Paget's disease of bone	Devoid of osteoclasts	No□a critical cytokine in the differentiation and activation of osteoclast	-	62

αvβ3 integrin, which binds to a variety of extracellular matrix proteins including vitronectin, osteopontin and bone sialoprotein. Several signaling and adaptor molecules were found to be involved in αvβ3 integrin-dependent signaling pathways, including phosphatidylinositol 3-kinase, c-Src, PYK2 and p130(cas). In addition, cytoskeletal molecules such as paxillin, vinculin, gelsolin and F-actin are recruited to adhesion contacts upon integrin activation. Many of these signaling and cytoskeletal molecules localize to the sealing zone of actively resorbing osteoclasts, suggesting that they play a role in linking the adhesion of osteoclasts to the bone matrix with the cytoskeletal organization, and the polarization and activation of these cells for bone resorption (103).

4.2. Mouse models

Our search of the English literature uncovered at least ten different animal models of OP each representing a unique gene mutation (Table 1)

4.2.1. Defects in *Atp6i*

The early arOP phenotype identified suggested a defect in osteoclast function resembling the naturally occurring *oc/oc* (osteosclerotic) mutation in mice, which exhibited defective resorption of osteoclasts as described by Marks *et al.* (104). These authors localized the murine *oc* mutation as a 1.6-deletion in the proximal region of chromosome 19 in the Atp6i gene (104). Following the isolation and characterization of the *ATP6i* gene in our laboratory (46;83;84), we proceeded to investigate the role of *ATP6i* in osteoclastic bone resorption through the generation of knockout mice with null mutation in ATP6 (46). Our results showed a severe osteopetrosis phenotype in the *ATP6i*$^{-/-}$ mice (Figure 2), akin to the human arOP, due to a defect of osteoclast-mediated extracellular acidification (Figure 3) (46). The defect in proton transport appeared to be limited to osteoclasts since these mice apparently retained normal pH in liver lysosome and proton transports in the kidneys. It has been reported that the osteopetrosis in *oc/oc* mouse is not amenable to hemopoietic stem cell transplantation (105); a counterintuitive result considering that the defect is intrinsic to the osteoclast, and probably due to the failure of these animals to engraft (50).

The osteoclast-specific expression of *ATP6i* and the osteopetrotic phenotypes in *ATP6i* knockout model suggested to us that the binding sites of genes important in osteoclast differentiation might exist in the promoter region of *ATP6i.* Interestingly, among AP-1, PU.1, and NF-kB, only PU.1 binding site was found in the 4-kb promoter region of *ATP6I* (83). In addition, several putative regulatory *cis* elements for Ets1, C/EBP, AP-2, H-APF-1, AP-3, PEA1, and PEA3 were observed (83). On-going efforts are directed towards characterizing the functions of these putative regulatory elements in controlling *ATP6i* gene expression.

4.2.2. Defects in *Clc-7*

Contrasting with other mouse models of osteopetrosis, *clcn7*$^{-/-}$ mice generate normal levels of osteoclasts capable of attachment to bone (29;48;85). However, these osteoclasts exhibited functional and morphologic abnormalities, including the inability to degrade bone in a pit assay (29). This is because of the inability of *clcn7*$^{-/-}$ osteoclasts to acidify the sealed extracellular space between bone and osteoclast (29). In addition, ruffled membranes were poorly developed in *clcn7*$^{-/-}$ osteoclasts. This is similar to an earlier observation reported in relation to osteoclasts from *oc*-mice that lack V-H^+ATPase subunit (29;85). Lack of ruffled border may correlate with a defective acidification of the resorption lacuna due either to a direct effect of pH in the lacuna, or to a decreased exocytotic delivery of membrane into the ruffled border (29). However, bone-facing areas of *clcn7*$^{-/-}$ osteoclasts contained V-H^+-ATPase, indicating that the biogenesis of this membrane by vesicle fusion is not entirely inhibited (29). Taken together, these findings suggest that osteopetrosis in *clcn7*$^{-/-}$ mice result from defective bone resorption due to failure of osteoclasts to secret Cl^- (29) and malignant osteopetrosis in both humans and the animal models is genetically heterogeneous and

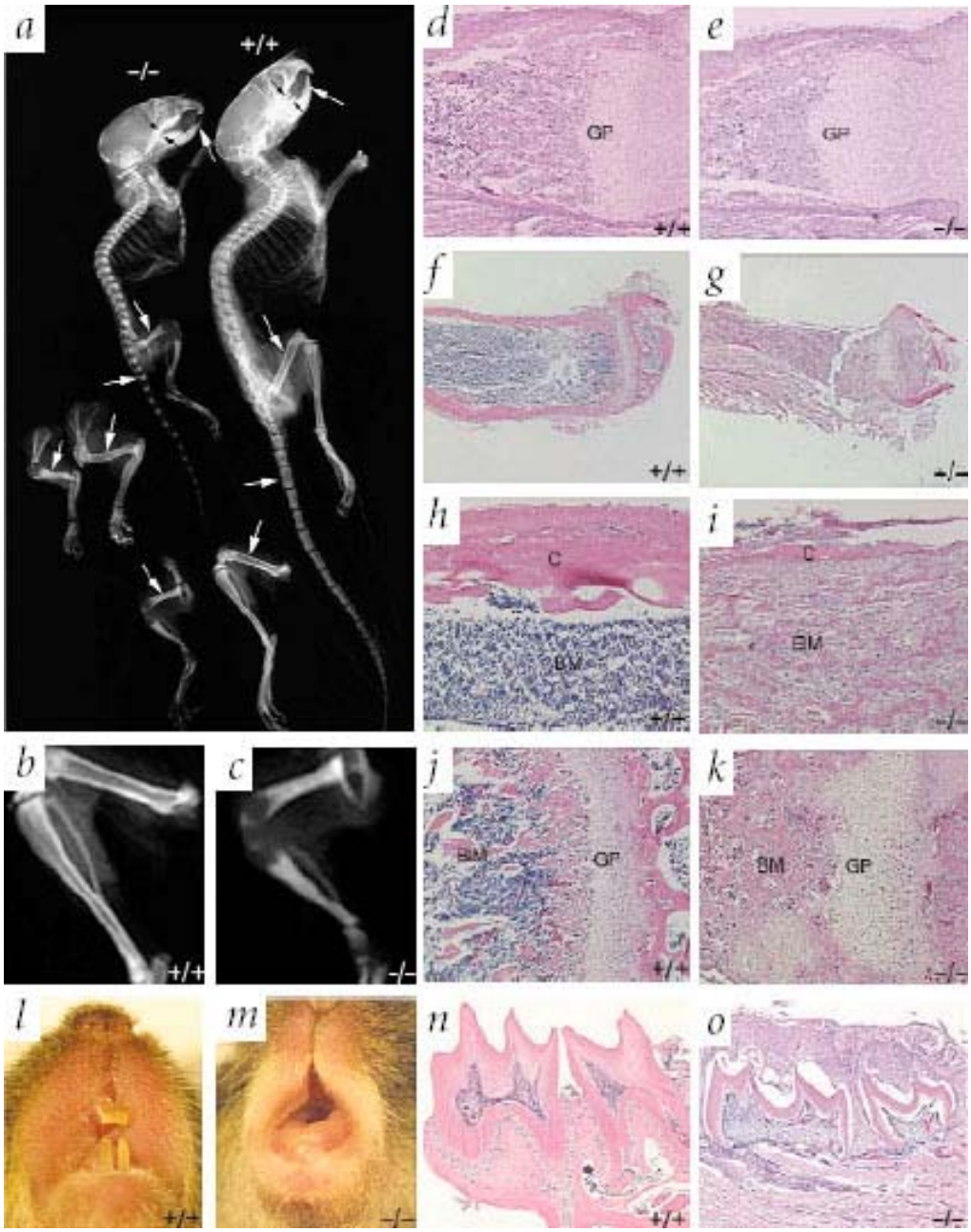

Figure 2. Radiographic and histological analysis of *Atp6i*$^{-/-}$ mice. *a*, Representative radiographs of the axial skeleton and limbs of four-week-old A*tp6i*$^{-/-}$ mice. *Atp6i*$^{-/-}$ mice have shortened limbs (arrows) compared with wild-type and *Atp6i*$^{+/-}$ littermates. There is sclerosis of the tibia and caudal vertebrae in *Atp6i*$^{-/-}$ mice (arrows). Mandibular and maxillary incisors and molars are not present in *Atp6i*$^{-/-}$ mice (arrows). The increased density in *Atp6i*$^{-/-}$ is uniformly present in all bones. *b,c*, High magnification of corresponding limbs in (*a*). *d-k*, Histological analysis of bone in wild-type (*d,f,h,j*)and *Atp6i*$^{-/-}$ littermates (*e,g,I,k*). *h,j*, High magnification of (*f*). *i,k*, High magnification of (*g*). The growth plates of *Atp6i*$^{-/-}$ mice at one day (*e*) and four weeks (*k*) had an extended and irregular zone of calcified cartilage compared with wild-type controls (*d,j*). In addition, the zone of the hypertrophic chondrocytes was increased in mutant mice (*e,k*). Note the abundance of calcified trabeculae present in the bone marrow cavity of four-week-old mutant mice leading to a lack of normal marrow cavity (*i*) compared with that of wild-type mice (*h*). The cortical bone in *Atp6i*$^{-/-}$ mice (*i*) is thin compared with that of wild-type littermates (*h*). A fracture was present in *Atp6i*$^{-/-}$ tibia (*g*). *i,m*, Photographs of incisor eruption in three-week-old mice. Incisor appeared in the oral cavity in normal mice (*l*), but did not erupt in *Atp6i*$^{-/-}$ mice (*m*). Histological analysis of teeth in wild type (*n*) and *Atp6i*$^{-/-}$ (*o*) littermates is shown. A section through the maxillary molar shows complete tooth eruption in three-week-old wild-type mice (*n*), whereas the teeth in mutant mice fail to erupt because of the inability of bone to resorb on the path of eruption (*o*). GP, growth plate; BM, bone marrow; C, cortical bone. Reproduced with permission from *Nature Genetics* (46).

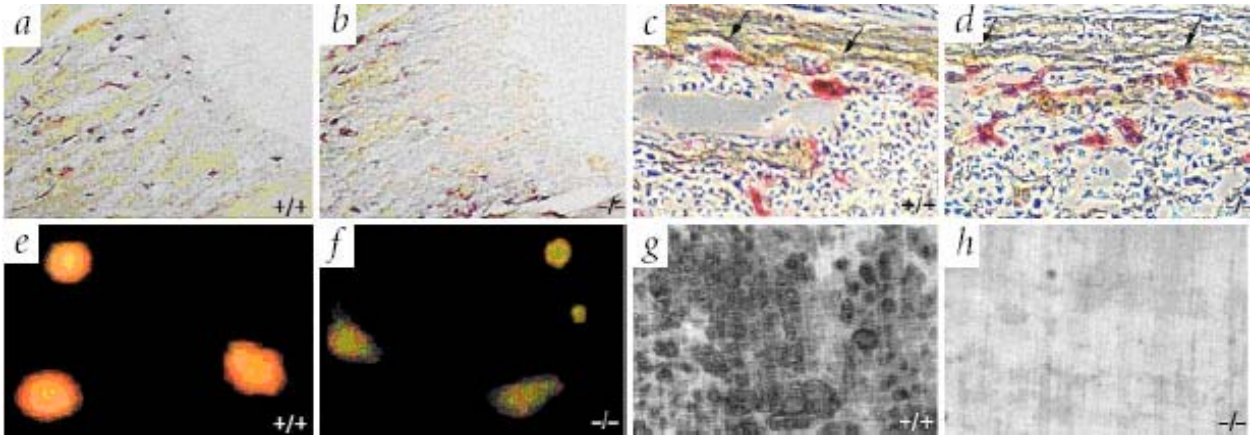

Figure 3. The morphology and properties of wild-type and *Atp6i*$^{-/-}$ osteoclasts. TRAP staining of one-day-old wild-type (a) and *Atp6i*$^{-/-}$ (b) tibia is shown. There are no quantitative or morphologic differences between wildtype and *Atp6i*$^{-/-}$ osteoclasts. c, High magnification of (**a**). **d**, High magnification of (**b**). *Atp6i*$^{-/-}$ osteoclasts attached to bone but did not form lacunae (**d**, arrows), whereas wild-type osteoclasts formed lacunae (**c**, arrows). **e,f,** Analysis of extracellular acid compartment formation. Acid compartments were formed as revealed by vital staining with acridine orange. Large, fluorescent orange discs were seen in wild-type OCLs (**e**), but absent from the *Atp6i*$^{-/-}$ OCL (**f**) preparation. *Atp6i*$^{-/-}$ osteoclasts showed a punctate pattern of faint orange fluorescence (**f**). The large, fluorescent orange discs in osteoclasts were attributed to extracellular acidification. **g,h**, Dentin resorption assay. Wild-type OCLs and *Atp6i*$^{-/-}$ OCLs were applied to dentin slices and cultured for three days. The dentin slices were stained using an anti-collagen type I polyclonal antibody, which reacts with exposed collagen fibrils that are apparent in the base of the lacuna. Many of the resorption pits were visible in the wild-type OCL preparation (**g**), but were not seen in the *Atp6i*$^{-/-}$ OCL preparation (**h**). **i**, Comparison of dentin pit formation by wild-type OCLs and *Atp6i*$^{-/-}$ OCLs. Data are means±s.e. from three experiments (**i**). Reproduced with permission from *Nature Genetics* (46).

other genes affecting osteoclast function can produce this phenotype. As in the oc/oc$^{(-/-)}$ mice, the *clcn7*$^{-/-}$ mice are not correctable with bone marrow transplantation (50).

4.2.3. Defects in *grey lethal (gl)*

The *grey lethal* (*gl*) mutant is the spontaneous mouse model that most closely resembles the severe human arOP (52). As in humans functional rescue of the *gl/gl* phenotype can be achieved by bone marrow transplantation, thus providing evidence of a cell-autonomous defect (52). Osteopetrosis in homogyzygous *gl/gl* mice is fully penetrant, and death ensues consistently at 3-4 weeks of age. Rajapurohitam *et al.* (52) reported the histologic features associated with the homozygous animals as including an abundance of multinucleated ostoeclasts with the osteoclast precursors fully differentiated into osteoclast-like cells in culture on a consistent basis. The authors also noted that the *gl/gl* osteoclasts displayed a defective cytoskeletal organization and an underdeveloped ruffled border associated with a marked reduction in resorptive function. This phenotype was shown not to result from either a c-Src-or TRAF-6, or cathepsin K-deregulated expression (52).

4.2.4. Defects in Cathepsin K

Previous cathepsin K knockout mice failed to mimic pycnodysostosis phenotype, indicating that the insertional mutation is probably hypomorph (106;107). Recently, we have generated a new cathepsin K mutant mouse strain by deletion of the cathepsin K promoter and the first two exons. The phenotype of cathepsin K homozygous mutant (cathepsin K$^{-/-}$) mice strikingly mimics human pycnodystostosis syndrome, with growth retardation, osteopetrosis, skull dysplasia, tooth and jaw-bone abnormalities, and acroosteolysis of the distal phalanges. Osteoclast-like multinucleated cells (OCLs) derived from cathepsin K$^{-/-}$ spleen-stromal co-culture showed normal morphology and extracellular acidification capacity, indicating that cathepsin K null mutation may not affect osteoclast mediated solubilization of bone mineral (108). Transplantation of wild-type bone marrow cells rescues normal bone development in the cathepsin K$^{-/-}$ mice, indicating that the bone marrow is the cellular origin of the defects.

4.2.5. Defects in αvβ3 integrin

Mice lacking beta3 (β3) integrins are osteosclerotic because of dysfunctional osteoclasts (72). Bone marrow macrophages derived from these mutants differentiate *in vitro* into numerous osteoclasts, thus establishing that a αvβ3 is not necessary for osteoclast recruitment. β3-null mice contain 3.5-fold more osteoclasts than do heterozygotes; however, these mutant osteoclasts are dysfunctional in resorbing whale dentin *in vitro*. The resorptive defect in β3-deficient osteoclasts may reflect the absence of matrix-derived intracellular signals, since their cytoskeleton is distinctly abnormal and they fail to spread *in vitro* to form actin rings *ex vivo*, or to form normal ruffled membranes *in vivo*.

4.2.6. Defects in genes regulating osteoclast differentiation

Although the deletion of factors affecting osteoclast formation such as PU.1, MCSF, TRAF-6, c-Src, NF-kB, c-Fos in mice caused severe forms of osteopetrosis, comparable defects have not been reported in human

osteopetrosis**.** Tondravi *et al.*(10) reported that PU.1 knockout mice are osteopetrotic and devoid of osteoclasts and macrophages. Osteopetrotic (*op/op*) mutant mice with a mutation in mcsf gene exhibit congenital osteopetrosis due to a severe deficiency of osteoclasts. The total number of mononuclear phagocytes is extremely low in affected mice (109). Mice deficient in TRAF6 and c-fos are osteopetrotic with defects in bone remodeling due to impaired osteoclast function (110;111). The study of Iotsava *et al.* (112) indicated that mice lacking NF-kB1 and NF-kB2 in a double knockout developed osteopetrosis because of a defect in osteoclast differentiation. Earlier reports on genetically engineered osteopetrotic mice suggest that c-fos proto-oncogene, a component of the AP-1 complex, block osteoclast development at the point of divergence from the common CFU-GM precursor (110). These events occur before the expression of osteoclast genes that may include *ATP6i* and others (110).

5. SUMMARY AND PERSPECTIVES

The clinical forms of human osteopetrosis result from defects in osteoclast bone resrption. The heterogeneous spectrum of identified phenotypes is said to result from heterogeneity of genetic defects leading to osteoclast dysfunction. Defects in osteopetrosis may be intrinsic to either the osteoclast-monocyte lineage or to the mesenchymal cells that make up the microenvironment responsible for supporting osteoclast ontogeny and activation. Using animal models, the genetic defects of some osteopetrotic mutations have been identified. For example, altered production of colony-stimulating factor 1 (CSF-1), a growth factor that is required by the mononuclear phagocytic system including osteoclasts, results in osteopetrosis in *op* mice and *tl* rats. A mutation in human chromosome 1p21 region near the CSF-1 gene has been identified in kindred with the autosomal dominant variant. Also a human mutation has been identified in kindred with infantile malignant osteopetrosis in the region of chromosome 11q12-13: an area of conserved synteny with the murine osteosclerosis mutation.

A number of specific clinical entities occurring side by side with osteopetrosis in frequencies or fashions enabling associations to be proposed have been reported. These include human pycnodysostosis, Glanzmann's thrombasthenia, severe pulmonary hypertension, and two cases of X-linked osteoperosis occurring alongside lymphoedema, anhidroitic ectodermal dysplasia, immunodeficiency, and incontinentia pigmenti (OL-EDA-ID). However, it would appear that the frequencies of each of these occurrences remain too few at the moment to qualify these associations as syndromic.

The medical treatment of osteopetrotic patients aims to stimulate host osteoclast or to provide the patient with an alternative source of viable osteoclast. Other approaches such as splenectomy and the restriction of dietary calcium have resulted in transient improvements only. High doses of calcitrol (1,25-dihydroxy vitamin D) have been used to stimulate osteoclast formation and bone resorption with reported success in the reversal of certain osteopetrotic parameters (113;114). The use of steroidal agents, parathyroid hormones, and human interferon-y has been reported with varied success (113). In all it would appear however that the hope of effective long-term cure of osteopetrosis rests with bone marrow transplantation, particularly with regard to the malignant autosomal recessive types.

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