V-ATPases and osteoclasts: ambiguous future of V-ATPases inhibitors in osteoporosis

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Abstract

Vacuolar ATPases (V-ATPases) play a critical role in regulating extracellular acidification of osteoclasts and bone resorption. The deficiencies of subunit a3 and d2 of V-ATPases result in increased bone density in humans and mice. One of the traditional drug design strategies in treating osteoporosis is the use of subunit a3 inhibitor. Recent findings connect subunits H and G1 with decreased bone density. Given the controversial effects of ATPase subunits on bone density, there is a critical need to review the subunits of V-ATPase in osteoclasts and their functions in regulating osteoclasts and bone remodeling. In this review, we comprehensively address the following areas: information about all V-ATPase subunits and their isoforms; summary of V-ATPase subunits associated with human genetic diseases; V-ATPase subunits and osteopetrosis/osteoporosis; screening of all V-ATPase subunits variants in GEFOS data and in-house data; spectrum of V-ATPase subunits during osteoclastogenesis; direct and indirect roles of subunits of V-ATPases in osteoclasts; V-ATPase-associated signaling pathways in osteoclasts; interactions among V-ATPase subunits in osteoclasts; osteoclast-specific V-ATPase inhibitors; perspective of future inhibitors or activators targeting V-ATPase subunits in the treatment of osteoporosis.

Key words: V-ATPase, osteoclasts, osteoporosis, pH, inhibitor, signaling pathways

1. Introduction

Vacuolar ATPases (V-ATPases) are protein complexes that couple ATP hydrolysis to proton transport in intracellular compartments or across the plasma membrane. V-ATPases are important in maintaining the acidic environment of intracellular organelles, including secretory granules, endosomes, and lysosomes. The acidic intracellular environment is necessary for protein sorting, zymogen activation, and receptor-mediated endocytosis [1]. V-ATPases also control the extracellular acidification of osteoclasts, which is a key factor for bone resorption [2]. The V-ATPases-related regulation of extracellular acidification also exists in other tissues or cells such as kidney and metastatic cells [3-5].

V-ATPases are ubiquitously expressed in a variety of cell types and are considered the essential “housekeeping” enzymes in all eukaryotic cells; however, the specific functions of V-ATPases vary from cell to cell. Most cells only express a low level of V-ATPases to carry out housekeeping functions, but other cells, like osteoclasts, have abundant V-ATPases that control extracellular acidification and finally affect bone resorption and bone remodeling. In this review, we will describe the V-ATPases-involved bone phenotypes, the functions of V-ATPase subunits in osteoclasts, as well as the inhibitors targeting V-ATPases in the treatment of bone diseases.

2. Structure of V-ATPase

The mammalian V-ATPase proton pump is
composed of the peripheral $V_1$ component and membrane-bound $V_0$ component with at least 13 subunits [6]. The $V_1$ component drives ATP hydrolysis to energize and initiate the rotation of $V_0$ domain, which includes eight subunits, $A$ through $H$. $V_0$ domain utilizes the energy generated by $V_1$ domain to translocate protons across the membrane and includes a through $e$ subunits. V-ATPases also have two accessory subunits, $Ap$1 and $Ap$2 [1, 7-10]. Some V-ATPase subunits have multiple isoforms, which are expressed or function in a tissue-specific manner [9, 11-13] (Table 1). N-glycosylation and other processes are crucial for the formation of subunit isoforms and protein stability [14, 15].

**Table 1. Subunits of human V-ATPases**

| Location | Name | Isoform(s) | Official Symbol | Location (GRCh38.p7) | mRNA and Protein(s) | Alias | Protein |
|----------|------|------------|----------------|----------------------|---------------------|-------|---------|
| $V_1$    | A    | ATP6V1A    | ATP6V1A        | Chromosome 3, NC_00003.12 (11374019,1138012058) | NM_001690.3 → NP_001681.2 | ATP6V1A, ARCL2D, HC66, VA68 | ATP6V1A, ARCL2D, HC66, VA68 |
|          | B    | ATP6V1B1   | ATP6V1B1       | Chromosome 2, NC_00002.12 (7093568,70965341) | kidney isoform: NM_001692.3 → NP_001683.2 | ATP6V1B1, ATP6B1 | ATP6V1B1, ATP6B1 |
|          | B2   | ATP6V1B2   | ATP6V1B2       | Chromosome 6, NC_00008.11 (20197139,20226832) | brain isoform: NM_001693.3 → NP_001684.2 | ATP6V1B2, HOS7 | ATP6V1B2, HOS7 |
|          | C    | ATP6V1C1   | ATP6V1C1       | Chromosome 8, NC_00008.11 (10032010,100370357) | NM_001695.4 → NP_001686.1 | ATP6V1C1, ATP6C, ATP6D | ATP6V1C1, ATP6C, ATP6D |
|          | C2   | ATP6V1C2   | ATP6V1C2       | Chromosome 2, NC_00002.12 (10720973,10785110) | isoform a: NM_001039362.1 → NP_001034451.1 isoform b: NM_004838.3 → NP_653184.2 | ATP6V1C2, ATP6C2 | ATP6V1C2, ATP6C2 |
| D        | D    | ATP6V1D    | ATP6V1D        | Chromosome 14, NC_000014.9 (67337864,67360003, complement) | NM_00594.3 → NP_005707.1 | ATP6V1D, ATP6M | ATP6V1D, ATP6M |
| E        | E1   | ATP6V1E1   | ATP6V1E1       | Chromosome 22, NC_00002.11 (17592136,17628822, complement) | isoform a: NM_001696.3 → NP_001687.1 isoform b: NM_001039366.1 → NP_001034451.1 isoform c: NM_00103967.1 → NP_001034456.1 | ATP6E6, ATP6E2, ATP6V1E1, ATP6V1E1 | ATP6E6, ATP6E2, ATP6V1E1, ATP6V1E1 |
|          | E2   | ATP6V1E2   | ATP6V1E2       | Chromosome 2, NC_00002.12 (4651185,46542557, complement) | isoform a: NM_001697.3 → NP_001687.1 | ATP6V1E2, ATP6E1, ATP6E2, ATP6V1E1 | ATP6V1E2, ATP6E1, ATP6E2, ATP6V1E1 |
|          | F    | ATP6V1F    | ATP6V1F        | Chromosome 7, NC_00007.14 (128862803,128865849) | isoform 1: NM_002421.3 → NP_002422.2 isoform 2: NM_00198909.1 → NP_00185838.1 | ATP6V1F, ATP6S14 | ATP6V1F, ATP6S14 |
| G        | G1   | ATP6V1G1   | ATP6V1G1       | Chromosome 9, NC_00009.12 (114587714,114988872) | NM_004888.3 → NP_004879.1 | ATP6V1G1, ATP6G, ATP6G1, ATP6G2, ATP6G2 | ATP6V1G1, ATP6G, ATP6G1, ATP6G2, ATP6G2 |
|          | G2   | ATP6V1G2   | ATP6V1G2       | Chromosome 6, NC_00006.12 (51544451,51564648, complement) | isoform a (longest): NM_130463.3 → NP_569730.1 isoform b: NM_130882.2 → NP_612139.1 isoform c: NM_00124078.1 → NP_001191007.1 isoform d: NM_133326.2 → NP_573569.1 isoform e: NM_133326.1 → NP_579872.1 isoform f: NM_001520218.1 → NP_001307146.1 isoform 1: NM_013914.1 → NP_057025.2 NM_21360.2 → NP_998785.1. Two variants encode the same isoform 1 isoform 2: NM_213619.2 → NP_998784.1 | ATP6V1G3, ATP6G3 | ATP6V1G3, ATP6G3 |
|          | G3   | ATP6V1G3   | ATP6V1G3       | Chromosome 1, NC_00001.11 (19852222,198540945, complement) | isoform a: NM_001300020.1 → NP_001123492.1 isoform b: NM_00130021.1 → NP_001123493.1 isoform c: NM_00251377.3 → NP_005168.2 | ATP6V1A1, ATP6N1, ATP6N1A | ATP6V1A1, ATP6N1, ATP6N1A |
| H        | H    | ATP6V1H    | ATP6V1H        | Chromosome 8, NC_00008.11 (53719543,53843111, complement) | isoform 1: NM_013914.1 → NP_057025.2 NM_21360.2 → NP_998785.1. Two variants encode the same isoform 1 isoform 2: NM_213619.2 → NP_998784.1 | ATP6V1H, CGI11, SFD | ATP6V1H, CGI11, SFD |
| V0       | a    | ATP6V0A1   | ATP6V0A1       | Chromosome 17, NC_000017.11 (42458844,42522579) | isoform a: NM_001130020.1 → NP_001123492.1 isoform b: NM_00130021.1 → NP_001123493.1 isoform c: NM_00251377.3 → NP_005168.2 | ATP6V0A1, ATP6N1, ATP6N1A | ATP6V0A1, ATP6N1, ATP6N1A |
|          | a2   | ATP6V0A2   | ATP6V0A2       | Chromosome 12, NC_000012.12 (123712318,123767155) | isoform a (OC116): NM_006019.3 → NP_006010.2 isoform b (TCIRG7): NM_006035.3 → NP_006544.1 isoform c: NM_001351059.1 → NP_00137988.1 | ATP6V0A2, ARCL2A, ATP6A2 | ATP6V0A2, ARCL2A, ATP6A2 |
|          | a3   | TCIRG1     | TCIRG1         | Chromosome 11, NC_000011.10 (6803995,68053846) | isoform a (OC116): NM_006019.3 → NP_006010.2 isoform b (TCIRG7): NM_006035.3 → NP_006544.1 isoform c: NM_001351059.1 → NP_00137988.1 | ATP6V0A2, ARCL2A, ATP6A2 | ATP6V0A2, ARCL2A, ATP6A2 |
|          | a4   | ATP6V0A4   | ATP6V0A4       | Chromosome 7, NC_000007.14 (6029032,6056832) | isoform a: NM_026022.2 → NP_005683.2 | ATP6V0A4, ATP6N1B | ATP6V0A4, ATP6N1B |
As shown in Figure 1, depending on ATP hydrolysis and reversible assembly process, the V-ATPase structure can be further divided into four parts: hexameric ring, central stalk, peripheral stalk, and proteolipid ring. The subunits A and B in the V1 component have three copies and they form an A3B3 hexameric ring in which ATP hydrolysis occurs. Three ATP catalytic sites are at the interfaces between subunits A and B [1]. Subunit A provides most of the residues for ATP binding. Other ATP binding sites may be located in subunit B [16-19]. The central stalk including subunit D and F bridges and stabilizes the interaction between V1 and V0 domains and couples the energy released from A3B3 to proton translocation in V0 [20, 21]. Subunit F is also crucial in ATP hydrolysis. The peripheral stalk, which contains three subunits A and B [1], functions as a stator and tethers the A3B3 hexameric ring to subunit a for the next rotational catalysis. The central rotor and peripheral stalk maintain the stability of V-ATPase complex during catalytic rotation and proton translocation [23-25]. Finally, subunit c and b in V0 form a proteolipid ring-like structure in the membrane layer [10, 26, 27]. Subunit d functions as a “boxing glove” on the top of the proteolipid ring, interacts with subunit a, and provides a connection between the central stalk of V1 and the proteolipid ring of V0 [1, 28, 29]. The proton transportation in V-ATPase relies much on the C-terminal domain of subunit a within the proteolipid subunits, in which the hemichannels allow protons to enter and leave the membrane [30-34]. The rotation of the central rotor initiates a series of proton translocations from subunit a to subunit c [35-37] (Figure 1). Subunits a3, d, A, C, and D are related to the coupling efficiency of ATP hydrolysis to proton transport, which alternatively regulates V-ATPase activity [38-42]. Besides ATP hydrolysis and proton transport, the reversible dissociation of V1 and V0 complexes also regulates V-ATPase functions [1, 43].

3. Subunits of V-ATPase and bone diseases

V-ATPase complex plays a significant role in biological and physiological processes. Mutations in the coding genes and non-coding regions of V-ATPase subunits cause various syndromes [44, 45]. The subunits of V-ATPase are ubiquitously expressed, and some of them have tissue or cell-specific distributions. Thus, the phenotypes of V-ATPase-related human diseases vary from the nervous system, kidney, and skin to skeletal system and many other tissues. Some subunits might contribute to common polygenic diseases, such as cancer and diabetes (Table 2). The two most striking and entirely distinct types of bone diseases that involve V-ATPases are osteopetrosis and osteoporosis.
Figure 1. Schematic map of V-ATPase. The mammalian V-ATPase proton pump is composed of peripheral V1 and membrane-bound V0. The V1 components include A-H subunits, whereas V0 includes a-e subunits. ATP hydrolysis and binding occur in the A3B3 hexameric ring. The central stalk D and F bridges and stabilizes V1/V0 interaction and couples the energy released from A3B3 to proton translocation in V0. The peripheral stalks E, G, H, C, and N-terminal of subunit a (NTa) function as stators and tether the A3B3 hexameric ring to subunit a. The proton transport relies on the C-terminal of subunit a (CTa) and proteolipid-containing b, c, d, and e.

Table 2. Subunits of V-ATPase and phenotypes in humans and animals.

| Gene Name | MIM Number | Human Disease Data | Mouse/Zebrafish Data |
|-----------|------------|--------------------|----------------------|
| ATP6V0A1 | N/A | N/A | Zebrafish: abnormalities in endosomes, autophagosomes, and phagolysosomes, as well as the migration of neural crest cells [46, 47]. |
| ATP6V0A2 | 219200 | Cutis laxa, autosomal recessive, type II A (ARCL-2A) [45, 48-51] | N/A |
| TCIRG1 | 278250 | Wrinkly skin syndrome [48-52] | N/A |
| ATP6V0A4 | 602722 | Renal tubular acidosis, distal, autosomal recessive [58, 59] | Mouse: distal renal tubular acidosis with hearing loss, severe metabolic acidosis, hypokalemia, early nephrocalcinosis, and bone loss [60, 61]. |
| ATP6V0B | N/A | N/A | Zebrafish: abnormal integument colorless, retina degeneration, and eye discoloration [62]. |
| ATP6V0C | N/A | Eye development and maintenance [63]; glial cell death/cancer/dopamine release/neurodegenerative disease [64, 65] | Zebrafish: abnormalities in head size, surface structure quality, fin malformation, pigment cell quality, brain necrosis, retinal pigmented epithelium quality, melanocyte quality, pectoral fin quality, nervous system quality [63, 66, 67]. |
| ATP6V0D1 | N/A | N/A | Zebrafish: manifestation in animal organ development, eye development, multicellular organism development, pigmentation, sensory organ development [63, 68, 69]. |
| ATP6V0D2 | N/A | N/A | Mouse: increased bone intensity [70, 71]. |
| ATP6V0E1 | N/A | N/A | N/A |
| ATP6V0E2 | N/A | N/A | N/A |
| ATP6V1A | 617403 | Autosomal recessive cutis laxa type IID [73] | Zebrafish: several abnormalities including suppression of acid-secretion from skin, growth retardation, trunk deformation [74]. |
| ATP6V1B1 | 267300 | Renal tubular acidosis with deafness [58, 75] | Mouse: acidosis, tubular, renal, with progressive nerve deafness [76]. |
| ATP6V1B2 | 134480 | Deafness, congenital, with onychodystrophy, autosomal | Mouse: hearing loss [77]. |
neutropenia. In animal studies, mice deficient in related to autosomal dominant severe congenital protein product and likely represent null alleles and mutations, may cause severe abnormalities in the insertions, nonsense substitutions, and splice site patients. The [91]. Transgenic mice carrying a dominant missense Tcirg1 pump activity. Deletion of the 5-prime portion of Tcirg1 89]. The mutations in [44, 53, 88] and infantile malignant autosomal recessive osteopetrosis (ADO II). ARO has a fatal outcome within the first decade of life. Besides ARO, TCIIRG1 mutations are also related to autosomal dominant severe congenital neutropenia [90]. In animal studies, mice deficient in Tcirg1 (Atp6i) show severe osteopetrosis. Atp6i/ osteoclast-like cells lose the function of extracellular acidification but retain intracellular lysosomal proton pump activity [57]. Deletion of the 5-prime portion of Tcirg1 gene in mice causes hypocalcemia and osteopetrorickets phenotype with high bone mass [91]. Transgenic mice carrying a dominant missense mutation (R740S) in Tcirg1 gene also exhibit high bone density without affected osteoblast parameters [55].

3.1 Subunits of V-ATPase and osteopetrosis
Osteopetrosis is a rare bone disorder with increased bone density. In the OMIM catalogue, autosomal recessive osteopetrosis is divided into seven subtypes (osteopetrosis, autosomal recessive 1~7, OPTB1~7; OMIM 259700, 259710, 259730, 611490, 259720, 611497 and 612301) while autosomal dominant osteopetrosis is divided into two types (osteopetrosis, autosomal dominant 1~2, OPTA1~2; OMIM166600, 607634). The clinical phenotypes of osteopetrosis vary considerably from the early onset life-threatening severe cases to mild cases in which the patients usually do not realize their conditions. Generally, osteopetrosis is clinically divided into three groups, i.e., infantile malignant autosomal recessive osteopetrosis (ARO), intermediate autosomal recessive osteopetrosis (IARO) and autosomal dominant osteopetrosis (ADO II). ARO has a fatal outcome within the first decade of life.

T cell immune regulator 1 (TCIRG1) encodes subunit a3 of V-ATPase and its mutations are the primary cause of autosomal recessive osteopetrosis [44, 53, 88] and infantile malignant osteopetrosis [54, 89]. The mutations in TCIRG1 underlie 50% of ARO patients [53]. The TCIRG1 variants include deletions, insertions, nonsense substitutions, and splice site mutations, may cause severe abnormalities in the protein product and likely represent null alleles [44, 53, 88]. Besides ARO, TCIRG1 mutations are also related to autosomal dominant severe congenital neutropenia [90]. In animal studies, mice deficient in Tcirg1 (Atp6i) show severe osteopetrosis. Atp6i/ osteoclast-like cells lose the function of extracellular acidification but retain intracellular lysosomal proton pump activity [57]. Deletion of the 5-prime portion of Tcirg1 gene in mice causes hypocalcemia and osteopetrorickets phenotype with high bone mass [91]. Transgenic mice carrying a dominant missense mutation (R740S) in Tcirg1 gene also exhibit high bone density without affected osteoblast parameters [55].

Besides subunit a3, so far, no other V-ATPase subunits have been reported to be involved in osteopetrosis. Only Atp6v0d2-deficient mice show increased bone mass. Subunit d2 has been suggested to play important roles in coupling proton transport and ATP hydrolysis as well as the assembly of ATPase complexes [39, 40]. Subunit d2 is also involved in the regulation of osteoclast function and bone formation. Although mutations in the human ATP6V0D2 gene have not been reported in osteopetrosis, Atp6v0d2 gene-knockout mice have increased bone density and defective osteoclasts because of the requirement for fusion of preosteoclasts resulting in osteopetrosis [70, 71, 92]. ATP6V0D2 has recently been identified as a novel chondrocyte hypertrophy-associated gene [93].

3.2 Subunits of V-ATPase and osteoporosis or bone loss
Osteoporosis is a common metabolic bone disease that is characterized by reduced bone mineral density (BMD) and increased risk of osteoporotic fractures. In particular, genes involved in the functions of osteoclasts have been associated with the risk of osteoporosis [94-96].

H subunit is a small subunit of V-ATPases that connects the V1 and V0 domains. We previously reported that partial loss of ATP6V1H function resulted in osteoporosis/osteopenia in a population of 1625 Han Chinese as well as in an Italian pedigree [86, 87]. Atp6v1h/ knockout mice generated by the CRISPR/Cas9 technique had decreased bone remodeling and a net bone matrix loss. Similarly, Atp6v1h/ osteoclasts showed impaired bone formation and resorption activity. The increased intracellular pH of Atp6v1h/ osteoclasts downregulated TGF-β1 activation, thereby reducing induction of osteoblast formation [86]. In a CRISPR/Cas9 zebrafish model, atp6v1h deficiency also caused bone loss [86, 87]. In another bivariate GWAS study, ATP6V1G1 was implicated as a novel pleiotropic gene affecting human BMD [84]. The above controversial effects of V-ATPase subunits on BMD suggest that the

| Subunit | Human | Mouse | Zebrafish |
|---------|--------|-------|-----------|
| ATPI6V1D1 | N/A | N/A | N/A |
| ATPI6V1C1 | N/A | N/A | N/A |
| ATPI6V1C2 | 609946 | N/A | N/A |
| ATPI6V1C1D | N/A | N/A | N/A |
| ATPI6V1E1 | 617403 | N/A | N/A |
| ATPI6V1E2 | N/A | N/A | N/A |
| ATPI6V1F | N/A | N/A | N/A |
| ATPI6V1G1 | N/A | N/A | Bone loss [84] |
| ATPI6V1G2 | N/A | N/A | Mouse: no obvious phenotype due to compensating increased G1 level [85]. |
| ATPI6V1G3 | N/A | N/A | N/A |
| ATPI6V1H | N/A | Bone loss [86, 87] | Mouse and zebrafish: bone loss [86, 87] |

N/A: no available data.
deficiency of V-ATPase subunits might not always lead to increased bone mass.

### 3.3 Other subunits of V-ATPase related to BMD

#### 3.3.1 Genetic factors for osteoporosis (GEFOS) information

GEFOS Consortium is a large international collaboration of groups studying the genetics of osteoporosis using the meta-analysis of GWAS data with high-density SNP arrays. The BMD of GEFOS was measured at the femoral neck and lumbar spine using dual-energy X-ray absorptiometry in 32,961 subjects (http://www.gefos.org/?q=content/data-release) [97]. We screened the variants of V-ATPase subunits in GEFOS data and in-house data to find whether more subunits are involved in BMD regulation. Based on our VEGAS analysis results of 2012 GEFOS-released data [98, 99], ATP6V1A, ATP6V0A1, ATP6V1E2, ATP6V0A4, ATP6V1F, ATP6V1G2 and ATP6V1G3 are related to BMD (Table 3). Further analysis showed that SNPs in other subunits of V-ATPase are also associated with BMD (Table S1). A detailed analysis of the GWAS data of 1627 Han Chinese [100, 101] revealed that other SNPs of V-ATPase subunits might be related to BMD (Table S2).

#### 3.3.2 Subunits of V-ATPase possibly related to BMD

Not much information is available on the location and functions of the above BMD-related subunits (A, E2, G2, G3, a1 and a4) of V-ATPase in osteoclasts.

**Subunit A:** The mRNA level of *Atp6v1a* has been reported in rat osteoclasts and may respond to fluid shear stress changes [102]. No other information about subunit A in osteoclastic function is available. The mutations in *ATP6V1A* gene caused autosomal recessive cutis laxa type IID (ARCL2D) [73]. Subunit A interacts with the N terminal of Wolfram syndrome 1 (WS1) protein in human embryonic kidney (HEK) 293 cells and human neuroblastoma cells, which might be important both for pump assembly in the endoplasmic reticulum (ER) and for granular acidification [103]. ATP6V1A also controls the extracellular acidification of intercalated cells in kidney, and its phosphorylation is regulated by the metabolic sensor AMP-activated protein kinase (AMPK) at Ser 384 [104]. Subunit A has also been detected in intracellular structures such as trans-Golgi network (TGN) of principal cells and narrow/clear cells in the epididymis and vas deferens [105]. The morpholinos against *atp6v1a* in zebrafish result in several abnormalities including suppression of acid-secretion from the skin, growth retardation, trunk deformation, and loss of internal Ca2+ and Na2+[74].

**Subunit E2:** Unlike testis-specific subunit E1, subunit E2, the isoform of E1 shows a ubiquitous expression in spermatocytes and rat epididymis [83, 106]. Subunit E2 was found to be present in the perinuclear compartments of spermatocytes and rat epididymis [83, 105].

### Table 3. Association of V-ATPase subunits and bone mass in GEFOS.

| Chr | Gene name | rSNP | Start Position | Stop Position | FNK% P value | SPN% P value |
|-----|-----------|------|----------------|---------------|--------------|--------------|
| 3   | ATP6V1A   | 30   | 113465865      | 113530905     | 0.00056      | 0.00042      |
| 2   | ATP7V1H   | 29   | 71162997       | 71192561      | 0.90179      | 0.204795     |
| 8   | ATP6V1B2  | 20   | 20054703       | 20079207      | 0.41588      | 0.675325     |
| 8   | ATP7V1C1  | 53   | 104033247      | 104085285     | 0.63862      | 0.917083     |
| 2   | ATP6V1C2  | 31   | 10861774       | 10925326      | 0.125874     | 0.386188     |
| 14  | ATP6V1D   | 17   | 67084580       | 6782720       | 0.411588     | 0.95045      |
| 17  | ATP6V1E1  | 44   | 18074902       | 18111586      | 0.198904     | 0.376264     |
| 14  | ATP6V1E2  | 10   | 46739885       | 46747096      | 0.282271     | 0.100897     |
| 8   | ATP6V1F   | 3    | 128502856      | 128505903     | 0.713287     | 0.40095      |
| 9   | ATP6V1G1  | 8    | 117349990      | 117361152     | 0.96004      | 0.812188     |
| 9   | ATP6V1G2  | 5    | 31512227       | 31514625      | 0.724276     | 0.148584     |
| 1   | ATP6V1G3  | 12   | 198492551      | 19850075      | 0.163836     | 0.017698     |
| 10  | ATP6V1H   | 62   | 54628102       | 54758871      | 0.08911      | 0.301698     |
| 17  | ATP6V1A1  | 12   | 40610861       | 40674597      | 0.448511     | 0.175924     |
| 12  | ATP6V1A2  | 35   | 12449664       | 124246301     | 0.797206     | 0.386216     |
| 7   | ATP6V1A4  | 86   | 138591028      | 138482941     | 0.025397     | 0.944056     |
| 1   | ATP6V0A8  | 4    | 44444061       | 44443972      | 0.94006      | 0.769231     |
| 1   | ATP6V0D1  | 12   | 67471916       | 67510890      | 0.664336     | 0.846154     |
| 9   | ATP6V0D2  | 93   | 87171138       | 87164545      | 0.204795     | 0.874126     |
| 7   | ATP6V0E1  | 21   | 172410762      | 172461900     | 0.183816     | 0.326673     |
| 7   | ATP6V0E2  | 3    | 149570056      | 149577801     | 0.657343     | 0.667333     |

*Chr*: chromosome; *Gene name*: gene symbol; *rSNP*: number of SNP; *Start Position*: position of the SNP on the chromosome; *Stop Position*: position of the SNP on the chromosome; *FNK% P value*: significance of the association of the gene with bone mass in GEFOS (femoral neck); *SPN% P value*: significance of the association of the gene with bone mass in GEFOS (spinal neck).
Subunit F: The mRNA level of subunit F has been reported in human prostate carcinoma cells and biopsy specimens from the antral mucosa [107, 108]. A zebrafish atp6v1f model was used to study its role during eye development. Atp6v1f mutant zebrafish showed oculocutaneous albinos and defects in melanosomes and retinal pigmented epithelium [63].

Subunit G2: Subunit G2 has a brain-specific distribution [109]. ATP6V1G2 was found to be associated with myocardial infarction according to analysis of SNPs and transcriptome sequencing [110, 111]. The mRNA of ATP6V1G2 was detected in lipopolysaccharide-stimulated human macrophages [112] and human neuroblastoma cell line SH-SY5Y [113].

Subunit G3: Subunit G3 is expressed in kidney and rat epididymis [105, 109]. Its protein is used as a novel immunohistochemical marker for differentiating subtypes of chromophobe renal cell carcinoma (RCC) including clear cell, papillary RCCs [114, 115], and osteosarcoma [116].

Subunits a1 and a4: Subunit a has four isoforms, a1-a4, among which subunit a3 plays an essential role in osteoclasts and bone density. Subunit a4 is expressed in α intercalated cells in both human and mouse kidney [117] as well as inner ear, olfactory epithelium, the uterus of pregnant animals, embryonic visceral yolk sac, prostatic alveoli, ampullary glands, epididymis, and vas deferens [118]. It is also associated with autosomal recessive distal renal tubular acidosis [58, 59, 109]. Atp6v0a4 knockout mice showed distal renal tubular acidosis with hearing loss, severe metabolic acidosis, hypokalemia, early nephrocalcinosis, and bone loss [60, 61]. Mutations in ATP6V0A4 were associated with atypical progressive sensorineural hearing loss in a Chinese patient with distal renal tubular acidosis [119]. A zebrafish study showed that rbcp3a and atrp6v0a1 promote endosomal maturation to coordinate Wnt signaling in neural crest [46].

3.4 Identification of subunits of V-ATPase involved in osteoclast formation

To further identify the relationship between osteoclasts and the subunits described above, we primary cultured mouse osteoclasts with induction by macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) and analyzed the mRNA levels of 13 V-ATPase subunits using RNA sequencing and Q-PCR. While we could not detect the expression of all subunit isoforms, at least one isoform was expressed representing each of the 13 V-ATPase subunits either by FPKM analysis or by Q-PCR analysis. Among the 15 expressed isoforms, nine (60%) showed an increased level of mRNA during osteoclastogenesis. Tcirg1 and Atp6v0a1 showed a time-dependent increase, and the other seven genes showed a sharp increase on day 4 and then dropped down on day 7, suggesting that subunits b, d2, A, B2, C1, D, and H contribute more to the early maturation stage of osteoclasts, and subunit a1 and a3 might play more important roles in late osteoclast maturation (Figure 2). Both RNA sequencing and Q-PCR analysis showed that the mRNA levels of subunits a4, e2, B1, C2, E2, G2, and G3 are quite low or undetectable in mature osteoclasts. In the above GEFOS analysis, ATP6V0A4, ATP6V1G2, and ATP6V1G3 are probably related to bone density. The low mRNA levels of a4, G2, and G3 in the mature mouse osteoclasts suggest that these subunits might affect bone density by their interactions with other subunits of osteoclasts or through a more complicated and systematic pathway instead of the osteoclast-specific pathway.

4. Functions of V-ATPase subunits in osteoclasts

The controversial effects of various subunits of V-ATPase on bone phenotypes imply their complicated biological functions in vivo and in vitro. While analyzing the bone phenotypes of various V-ATP subunits, we need to be cognizant of whether the subunits are specifically expressed in osteoclasts, the pathway(s) by which the subunits affect osteoclast function, and whether it is a common phenomenon or unique to a particular subunit. At present, there are only limited human or animal data on the relationships between subunits a3, d2, B2 [120, 121], C1 [122-126], and H and osteoclast functions. It is, therefore, difficult to conclude that a particular subunit is specifically expressed in osteoclasts and only regulates osteoclastic function. As per our mRNA screening of ATPase subunits in mouse osteoclasts, more subunits, such as b, c, e1, A, D, E1, F and G1, have potential functions in osteoclasts. Thus, it is necessary to consider the direct or indirect functions of V-ATPase subunits in osteoclasts and non-osteoclasts while exploring the mechanisms of V-ATPase-regulated bone phenotypes.

4.1 Direct roles

4.1.1 Regulation of extracellular acidification

V-ATPases complexes containing subunits a3, d2, and C1 are present on the ruffled borders of osteoclasts to maintain the acidic extracellular environment [127]. Subunit a3 of V-ATPase controls the extracellular acidification of bone resorptive lacunae [57]. Atp6v0a2 is highly expressed in mature mouse osteoclasts, and its depletion abolishes their
extracellular acidification [71]. Subunit C1 is also highly expressed in osteoclasts and interacts with the α3 subunit on the ruffled borders. Deficiency of C1 subunit causes severely impaired osteoclast acidification activity and bone resorption [122] (Figure 3A).

4.1.2 Maintenance of pH homeostasis of intracellular compartments

V-ATPases exist within intracellular compartments including endosomes and lysosomes. They control the acidification of these vesicles and regulate membrane trafficking processes such as receptor-mediated endocytosis, intracellular trafficking of lysosomal enzymes from Golgi complexes to lysosomes, protein processing and degradation, and transportation of small molecules and ions. The low pH in the intracellular compartments enables endocytosis of ligands or other proteins including low-density lipoprotein (LDL) and their dissociation from their receptors [128, 129]. ATP6V1H is localized in osteoclasts, and its deficiency causes an increase in intracellular pH and inhibition of the formation and function of osteoclasts such as bone resorption [86] (Figure 3A).

V-ATPase complex also facilitates the entry of many viruses, and endosomal acidification enables fusion of viruses with infected cells [130, 131]. Subunit H is a representative example that helps HIV internalization [132]. During infection of group A rotaviruses (RVAs), the outer capsid proteins of the RVA strain bind to cell surface receptors and phosphorylate PI3K, Akt, and ERK, which, in turn, directly interact with subunit E to acidify late endosomes for uncoating of RVAs [133]. Also, ATP6V0C is related to human cytomegalovirus (HCMV) [134].

4.1.3 Assembly of cytoskeletal F-actin

Subunit C1 regulates cytoskeletal F-actin assembly during osteoclast activation [123-125] and the reversible dissociation of V-ATPases [126]. Local treatment of AAV-shRNA-Atp6v1c1 attenuates the bone erosion and inflammation caused by periodontitis. Atp6v1c1 silencing severely impairs osteoclast acidification and bone resorption as well as F-actin ring formation, whereas cell differentiation does not appear to be affected [135] (Figure 3A).

Subunit B2, and not the B1 isoform, is located in intracellular vesicles and on ruffled membranes of osteoclasts [120, 121]. Phosphatidylinositol 3-kinase (PI 3-kinase) is involved with the association of B2 and F-actin that is important for recruitment of V-ATPase complexes to the osteoclasts’ ruffled border during polarization and bone resorption [136-139]. Alteration of B2 and F-actin association by the mutated binding site does not influence V-ATPase assembly or ATP hydrolysis [138, 139].
Figure 3. Functions of V-ATPases in osteoclasts. (A) Direct regulation of extracellular and intracellular pH. V-ATPases on ruffled borders maintain the acidic extracellular environment for bone resorption. V-ATPases in endosomes and lysosomes keep pH homeostasis of intracellular compartments. V-ATPases are associated with cytoskeletal F-actin. (B) V-ATPases are required for the fusion of preosteoclasts. (C) Indirect roles in osteoclasts. V-ATPases regulate endocytosis, vesicle trafficking, protein processing, and degradation as well as enzyme secretion and enzymatic activities.

4.1.4 Fusion of preosteoclasts
Subunit d2 is required for the fusion of preosteoclasts. Atp6v0d2-deficient mice have defective osteoclasts and increased bone mass [70, 71, 92] (Figure 3B).

4.2 Indirect roles
4.2.1 Effects on endocytosis and vesicle trafficking
V-ATPase complex functions as a proton pump and acidifies endosomes and lysosomes. V-ATPase-specific inhibitors or acidotropic agents may inhibit endosomal acidification, thus affecting endocytosis, vesicle trafficking, and the processing of secretory and lysosomal proteins [1, 141]. Atp6v0c-mutant mice show acidification defects in endocytosis and Golgi complex that affect the development of embryonic and extraembryonic tissues [142]. Subunit c and a2 interact with two small GTPases, Arf6 and ARNO, respectively; inhibition of these interactions causes an alteration in endocytosis. Recruitment of Arf6 and ARNO from the cytosol to endosomal membranes causes intra-endosomal acidification [143]. In yeast, regulator of the H+-ATPase of vacuoles and endosomes (RAVE) is essential for the reversible assembly of V-ATPase. RAVE complex consists of Rav1p, Rav2p, and Skp1p, which play an essential role in RAVE regulation of V-ATPase activity [144]. It is unclear whether V-ATPases promote membrane fusion in the endocytic and exocytic pathways independent of their acidification functions [145].

4.2.2 Effects on endoplasmic reticulum (ER)
Besides endosomes and lysosomes, V-ATPases are also localized in the ER. The functions of V-ATPases in the ER are related to protein processing and degradation. Mutation analysis shows that R444L causes subunit a3 to be stuck in the ER instead of lysosomes. The oligosaccharide moiety of the mutant protein is misprocessed, and its degradation is through the ER-associated degradation pathway. R445L mutated protein is also degraded quickly in differentiated osteoclasts due to its altered protein conformation [146]. Archazolid, a V-ATPase inhibitor, affects the secretion of cytokines TNF-α, interleukin-6, and -8, and causes accumulation of these cytokines at the ER [147].

4.2.3 Effects of V-ATPases on enzyme activity
There are several reports on V-ATPases and cathepsins. Cathepsin K (CTSK), an important enzyme for osteoclasts to resorb bone matrix, requires V-ATPase to keep an acidic environment for its normal activity [148]. Archazolid induces secretion of the pro-forms of cathepsin B and D. By inhibiting

Golgi-associated V-ATPase activity relies on actin and the Golgi pH. Actin depolymerization promotes dissociation of V1 and V0 domains followed by translocation of subunit B2 from Golgi membranes to the cytosol. Actin may regulate Golgi pH homeostasis, which can maintain the coupling of V1-V0 domains of V-ATPase through the binding of microfilaments to subunits B and C [140].
mannose-6-phosphate receptor-dependent trafficking, Archazolid abrogates the cathepsin B maturation process, reduces the intracellular mature cathepsin B protein abundance, and decreases cathepsin B activity [149].

Other enzymes related to V-ATPases include members of the matrix metalloproteinase (MMP) family. We recently observed that deficiency of subunit H affected MMP9 and MMP13 in the bone system [87]. In pancreatic cancer cells, V-ATPase colocalizes with cortactin, an F-actin-stabilizing protein, which helps in the release of MMPs. V-ATPase selectively modulates specific MMPs such as MMP9 and MMP2, which might be linked to an invasive cancer phenotype [150]. Treatment with concanamycin or Atp6v1e shRNA affects MMP9 and MMP2 activities. Targeting inhibition of α2 subunit suppresses the activities of MMP9 and MMP2 in ovarian cancer cells and plays a critical role in tumor invasion [151] (Figure 3C).

4.3 Signaling pathways in osteoclasts involving V-ATPases

Cellular signaling pathways play critical roles in cell-cell and cell-environment interactions and maintaining extrinsic and intrinsic homeostasis. The important association between V-ATPases and cellular signaling has been documented in cancer cell biology to affect apoptosis, tumor cell invasion, migration, and metastasis [128, 152]. Recent findings have shown that V-ATPases also affect cellular signaling pathways in osteoclasts and bone metabolism [86] (Figure 4A-C).

4.3.1 WNT/β-catenin signaling pathway

WNT signaling is initiated by the binding of WNT ligand to its receptor Frizzled (FZ), forming a complex with a specific co-receptor low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). WNT signaling includes β-catenin-dependent canonical and β-catenin-independent noncanonical signaling pathways. WNT signalings, such as Wnt3a and LRP5/6, have been shown to influence bone mass [153, 154] or inhibit osteoclast differentiation by activating canonical and noncanonical cAMP/PKA pathways [153-159]. Lack of LRP5/6 may impair osteoclast progenitor proliferation [155].

V-ATPases influence WNT signaling through its trafficking and activation pathway including blocking the phosphorylation of its receptor and its internalization after ligand binding. In addition, localization and activation of the WNT receptor require an adaptor (Pro)renin receptor (PRR), one of the accessory subunits of the V-ATPase complex. WNT secretion also requires its binding to the carrier protein Wntless (WLS), and V-ATPase-controlled vacuolar acidification facilitates the release of palmitoylated WNT3A from WLS in secretory vesicles [160].

4.3.2 Notch signaling pathway

Notch signaling connects the communication between signal-sending and signal-receiving cells. As
a single pass transmembrane receptor, Notch receptor includes extracellular and intracellular domains (NICD). The ligand binding to the extracellular domain of the Notch receptor activates its successive proteolytic cleavage and releases NICD, which then translocates to the nucleus and alters gene transcription, cell identity, and growth. Notch signaling affects osteoclastic differentiation, maturation, and its resorption activity. Both osteoclast precursors and bone stromal cells are regulated by Notch signaling [161-168]. Notch2 in osteoclasts controls bone resorption via PYK2-c-Src-microtubule signaling pathway [162].

The proper processing, trafficking, and activation of Notch signaling require V-ATPase participation [169]. The acidic environment of early endosomes enables S3 cleavage through secretase and NICD release in a V-ATPase-dependent manner [170]. Mutant V-ATPase affects the internalization of Notch signals and causes its accumulation in lysosomes as well as a substantial loss in the physiological and ectopic Notch activation of endosomes [170]. Rabconnectin-3 α and β (Rbcn-3A and B) regulate the V-ATPase proton pump and mutations in Rbcn-3A and Rbcn-3B cause defects in endocytic trafficking and the accumulation of Notch in late endosomal compartments in Drosophila [171].

4.3.3 mTOR signaling pathway

The mechanistic target of rapamycin (mTOR), the former mammalian target of rapamycin, is a member of phosphatidylinositol 3-kinase-related kinase family. mTORC1 is a central node of cellular signaling, and its activity is influenced by multiple factors such as growth factors, stress, energy status, and cellular amino acid levels, whereas mTORC2 is involved in survival signaling. The inhibition of V-ATPases impairs lysosomal acidification and alters lysosomal amino acid efflux, which also shows an mTOR-dependent regulation mechanism [172].

A novel polypeptide translated by long non-coding RNAs exists in the late endosome/lysosome and interacts with the lysosomal V-ATPase, which may negatively regulate mTORC1 activation by amino acid stimulation, rather than by growth factors [173, 174]. mTOR activity is essential for osteoclastic function and bone metabolism [175-177]. It regulates osteoclast formation by modulating the C/EBP-β isofrom ratio [177]. mTOR and AMPK function as the nutrient and energy sensors of osteoclasts and regulate osteoclastogenesis [176].

mTOR1 activity is dependent on V-ATPase function. V-ATPases sense the signal of amino acid sufficiency and transfer it to mTORC1. V-ATPases also control the off/on switch of the V-ATPase-Ragulator complex, which plays a vital role in the activation of AMPK in lysosomes [128]. Amino acid starvation strengthens the association of Ragulator with the V1 segment of V-ATPase instead of V0 [178]. mTOR undergoes both lysosomal and proteasomal degradation, which follows the guanine nucleotide exchange of Rag small GTP-binding protein activated by V-ATPase [179, 180]. In osteoclasts, mTOR activation and deactivation rely on the lysosomal environment. The direct evidence for the interaction between V-ATPase and mTOR1 in osteoclasts is that R740S mutation in subunit a3 alters mTOR1 expression and activity in osteoclasts [175].

4.3.4 GTP-binding protein-coupled receptors (GPCR) signaling pathway

GPCRs belong to a large family with seven transmembrane domains and constitute extracellular sensing components and intracellular signal transduction cascade. The representative GPCRs that regulate osteoclasts and bone metabolism include the parathyroid hormone receptor (PTHR) [181, 182]. V-ATPases regulate GPCRs through the modulation of intracellular pH. Acidification of intracellular components induces dissociation of PTH from PTHR and stimulates the signaling of activated receptors and their recycling [128]. PTH also induces the phosphorylation of subunit a via cAMP-dependent protein kinase (PKA), which positively regulates the catalytic activity of the pump [179]. In addition, V-ATPase-mediated endosomal acidification provides negative feedback on PTH signaling. Normal pH enables binding of arrestin, and lower endosomal pH causes enhanced retromer binding and turns off PTH receptor signaling [183].

5. Complex contributions of V-ATPase subunits to bone phenotypes

5.1 Interactions between V-ATPase subunits

The direct interactions between various subunits of V-ATPase have been studied for the past decades to clarify the assembly and mechanisms of V-ATPase complex using bioinformatic model systems, mutation analysis, binding experiments, etc. In the model of the V1 component of Thermus thermophilus V-ATPase, interactions between A3B3 and D/F subcomplexes were observed, and asymmetry was realized by rigid-body rearrangements of the relative position between A and B subunits [184].

Based on hybridization or pull-down analysis, subunit a3 is connected with subunits d2 or B2. The connection between a3 and B2 was found to be quite important for the trafficking of ruffled border
V-ATPase in activated osteoclasts [136] and this interaction was affected by benzoxyhydradize derivative IPI and KM91104 [3,4-dihydroxy-N-(2-hydroxybenzylidine) benzoxyhydradize] [185]. According to acryo-EM reconstruction model of yeast V-ATPase, when the V1 segment was released from V0, the N-terminal cytoplasmic domain of subunit a changed its conformation and bound to the rotor subunit d [186]. N-termini of subunits a3 and d2 had high-affinity interactions as found by glutathione-S-transferase (GST) pull-down assay, and were co-expressed in mammalian cells [71]. Luteolin inhibited V-ATPase activity by interfering with a3-d2 interaction without affecting the transcription or protein levels of these subunits [187-190], and Janus kinase (JAK) signaling pathway was involved in this process [191].

Using an RNA sequencing technique, the mRNA response of all V-ATPase subunits was observed in haploinsufficiency mouse osteoclasts (Atp6v1h+/−), and some subunits showed increased expression (Table 4). Subunit d2 showed a two-fold increase in expression, whereas other subunits spatially close to subunit H, such as subunits B2, C1, D, A, and G1, exhibited a varied level of increased expression to compensate for the subunit H haploinsufficiency. Subunit H has been regarded as a functional bridge between V1 and V0 and its absence resulted in an inactive V1/V0 complex by reducing communication between the two components [192]. Thus, subunit H might function as a negative regulator of osteoclastic formation and function, and the upregulated mRNA levels of the other V-ATPase subunits might not definitely lead to increased V-ATPase function.

5.2 Interactions between osteoclasts and osteoblasts

Although there is no direct evidence for V-ATPases in osteoblasts, increasing data imply some functions of V-ATPases in osteoblasts and bone formation. For example, deletion of the 5-prime portion of the Tcirg1 gene (subunit a3) in mice caused hypocalcemia and osteopetrosis phenotype combined with decreased bone formation [91]. Atp6v0d2-deficient mice had enhanced bone formation and osteopetrosis [70, 71, 92]. Atp6v1h+/− mice showed a decrease in cartilage and bone formation with reduced bone formation rate and mineral apposition rate. The numbers of osteoblasts, the area of osteoblast surfaces as well as the osteoblast ALP level were reduced in Atp6v1h+/− mice [86]. ATP6V1H was recently detected in mouse bone marrow stromal cells and deficiency of ATP6V1H impaired their osteogenic differentiation and enhanced adiogenic differentiation[193].

### 5.3 Systemic factors

A variety of systemic factors may affect bone metabolism, including growth factors and hormones. Due to the ubiquitous distribution of V-ATPases, various subunits of V-ATPase may affect bone phenotypes in a general and complex fashion.

#### Table 4. Comparison of mRNA levels of V-ATPase subunits in mouse osteoclasts.a

| Gene Name   | Length | Atp6v1h+/− Expression | Atp6v1h+/− Expression | log2 Fold Change (Atp6v1h+/−/Atp6v1h+/−) | Padj | Up/ Down b | P value |
|-------------|--------|-----------------------|-----------------------|------------------------------------------|------|------------|---------|
| Atp6v1h2    | 2742   | 23955.49              | 42023.38              | 0.810836                                 | 1.24×10^-19 | -          | 2.02×10^-19 |
| Atp6v1d2    | 2518   | 4611.703              | 9576.583              | 1.04211                                  | 0.0014       | Up         | 5.98×10^-10 |
| Atp6v1c1    | 2117   | 4933.716              | 6902.68               | 0.49698                                 | 0.000456     | -          | 2.47×10^-4 |
| Atp6v1p2    | 2376   | 4700.42               | 7147.419              | 0.60433                                 | 0.008669     | -          | 0.00096 |
| Atp6v1d1    | 1410   | 4022.583              | 5443.382              | 0.43038                                 | 0.29975      | -          | 0.04732 |
| Atp6v1a1    | 3959   | 14607.46              | 19002.6               | 0.44625                                 | 0.056436     | -          | 0.00607 |
| Atp6v1g1    | 1104   | 11094.781             | 6122.023              | 0.323943                                | 0.393295     | -          | 0.00736 |
| Atp6v1b1    | 996    | 11082.52              | 14157.32              | 0.353262                                | 0.04682      | -          | 0.00845 |
| Atp6v1e1    | 800    | 5985.754              | 7517.969              | 0.32881                                 | 0.096908     | -          | 0.021782 |
| Atp6v1c1    | 1219   | 5479.907              | 6871.928              | 0.325563                                | 0.09754      | -          | 0.022242 |
| Tcirg1      | 2719   | 25921.58              | 31894.51              | 0.299134                                | 0.101496     | -          | 0.023429 |
| Atp6v1e1    | 1520   | 61788.39              | 77716.21              | 0.33088                                 | 0.157199     | -          | 0.035293 |
| Atp6v1f1    | 635    | 4880.556              | 5953.098              | 0.28708                                 | 0.208681     | -          | 0.063749 |
| Atp6v1g1    | 1632   | 59.3336               | 42.74398              | -0.47313                                | 0.412445     | -          | 0.169435 |
| Atp6v1e1    | 3997   | 8717.704              | 6991.545              | -0.31834                                | 0.53171      | -          | 0.249876 |
| Atp6v1d1    | 1617   | 10210.97              | 8998.879              | -0.1823                                 | 0.612442     | -          | 0.31818 |
| Atp6v1a2    | 5357   | 2573.471              | 5737.729              | -0.11656                                | 0.657228     | -          | 0.356739 |
| Atp6v1p1    | 2189   | 22026.99              | 19970.68              | -0.14139                                | 0.658755     | -          | 0.358188 |
| Atp6v1e1    | 1849   | 5.238299              | 3.990053              | -0.39269                                | 0.816266     | -          | 0.549267 |

a All data were generated by our group. Osteoclasts were primary cultured from wild-type and Atp6v1h+/−mice as previously reported. Total mRNA of cells was extracted after induction by M-CSF and RANKL for 7 days [86]. High-quality RNA was obtained and RNA sequencing analysis was performed. All coding genes of V-ATPase subunits were compared between two groups. N=3.

b Bold: Up / Down: log2-fold change >1 or -1 and P value <0.05; Bold font shows the mRNA changes with a significant P value (P < 0.05).

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5.3.1 Immune factors

Subunit a3 has two transcript variants, the longer isoform OC116 and the shorter isoform TIRC7. The short one uses a transcription start site within the exon 5 of the longer variant (OC116) and includes the downstream intron as part of its 5' UTR. TIRC7 is expressed in T lymphocytes and is essential for normal T cell activation. OC116 isoform encodes osteoclast-specific subunit a3, while TIRC7 mRNA is expressed by alloactivated T lymphocytes as a T cell inhibitory receptor [194, 195]. Six new alternative splice events in TCIRG1 were observed in 28 human tissues, implying that more functions of TCIRG1 might exist besides immune response and bone resorption [196].

5.3.2 Insulin and other hormone factors

Insulin activates ERK1/2 MAP kinase of osteoclasts and induces the expression of NFATc1 and Atp6v0d2. The insulin-induced expression of Atp6v0d2 was blocked by the ERK1/2 inhibitor or knockdown of insulin receptor [197]. The acidifying secretory vesicles in B-cells of pancreatic islets are the major site of proinsulin to insulin conversion. Subunit a3 is expressed in the endomembrane of secretory vesicles of normal islet B-cells but is absent in insulinoma cells, suggesting that subunit a3 may have a profound effect on the efficiency of proteolytic cleavage of proinsulin [198].

A mouse model with a null mutation at the subunit a3 locus exhibited a reduced level of insulin without changing the processing of insulin. In this respect, subunit a3 was believed to regulate the exocytosis process of insulin secretion [59]. V-ATPase functioned as a sensor of cytosolic pH, and was required for full activation of the cAMP-dependent PKA pathway in response to glucose in the Min6 β-cell line and contributed to insulin secretion [199]. Bafilomycin exposure also inactivated the insulin/IGF signaling pathway intermediate FOXO1 and increased the insulin content in neonatal islets [200].

The accessory subunits of V-ATPase such as AP1 (Ac45) and AP2 (PRR) are related to insulin. Ac45 was highly expressed in Langerhans cells of islets. Downregulation of Ac45 reduced insulin secretion and proinsulin II processing [201]. AP2 was abundant in islets including both α and β cells and modulated both glucagon-like peptide-1 receptor (GLP1R) and insulin processing to affect insulin secretion [202]. AP2 (PRR), another accessory protein of V-ATPase, was expressed in pituitary adenoma cells and regulated growth hormone (GH) release via V-ATPase-induced cellular acidification [203]. The PRR blocker could reduce body weight and fat mass and improve insulin sensitivity in high-fat-fed mice. Knocking out the PRR gene of adipose tissue also prevented weight gain and insulin resistance [204].

5.3.3 Growth factors

V-ATPases and specifically its subunits B2, c, and E were found to be involved in TGF-β1-mediated epithelial-to-mesenchymal transition (EMT) in rat proximal tubular epithelial cells (NRK52E) [205]. Since TGF-β1 activation is pH sensitive [206, 207] and TGF-β1 regulates the functions of osteoclasts and osteoblasts [208, 209], changes in the intracellular pH of Atp6v1h−/− osteoclasts altered the level and activity of TGF-β1, which further affected osteoblasts and osteoclasts [86]. Atp6v0a2-knockout mice showed delayed mammary morphogenesis associated with aberrant activation of Notch and TGF-β pathways [210].

The low pH in intracellular components was also found to be responsible for ligand dissociation and receptor trafficking of epidermal growth factor receptors (EGFRs) and insulin receptor. There were differential routings of internalized EGFRs induced by EGF, TGF α, and the superagonist EGF-TGF alpha chimera E4T. Bafilomycin treatment blocked EGFR but not c-Cbl degradation [211].

6. Future applications of V-ATPase inhibitors in osteoporosis

Osteoporosis is characterized by relatively increased bone resorption, and current osteoporosis therapeutics are mainly based on antiresorptive treatment [212, 213]. The anti-resorptive molecules antagonize integrin or inhibit Src tyrosine kinase, V-ATPases, chloride channels or cathepsin K. Bisphosphonates and Denosumab, the antibody against RANKL, are widely recommended as the first-line antiresorptive therapy [214]. Some of these have disadvantages such as coupling of inhibition of bone resorption and bone formation. Thus, the specificity of the antiresorptive therapy is essential.

As reviewed above, bone resorption requires V-ATPases activity and localization of some subunits of V-ATPase in osteoclasts. Successive generations of antiresorptive drugs based on V-ATPase complex or a specific subunit are becoming increasingly attractive. However, future efforts should focus on reducing side effects, minimizing the frequency of dosing, and increasing efficacy to halt osteoporotic bone loss [215-220].

6.1 V-ATPase inhibitors as anti-resorptives

6.1.1 Non-osteoclast-specific inhibitors of V-ATPase

The representative inhibitors of V-ATPase include bafilomycin A1 and B1, concanamycin A, and...
pleomacrolides. These highly specific V-ATPase inhibitors have been widely used in experimental cell biology for decades. Besides these inhibitors, macrolactone, archazolid, benzolactoneenamide and apicularen type inhibitors have also been shown to bind to the V0 component of V-ATPase. The agents are linked to the holoenzyme at or near the a/c subunit interface [213, 221, 222]. Many V-ATPase inhibitors show anti-tumor effects by targeting EMT or changing intracellular pH [223-226]. Other new inhibitors including FK506 might be possible new therapies for treating neurodegenerative diseases [227].

6.1.2 Osteoclasts-specific inhibitors of V-ATPase

The above inhibitors have broad effects and, therefore, many other osteoclasts-specific inhibitors of V-ATPases have become more attractive for osteoporosis treatment in recent decades [185, 191]. SB242784 is one of the attractive inhibitors with high potency and selectivity for the osteoclast V-ATPase [228]. Other inhibitors targeting osteoclastic V-ATPases include FR167356, FR202126, FR177995, Diphyllin, saliphenylhalamide (saliPhe) and lejimalides (IEJLs) [229-232]. These inhibitors are summarized as follows (Table 5).

**SB242784:** (2Z,4E)-5-(5,6-dichloro-2-indolyl)-2-methoxy-N-(1,2,2,6,6-pentamethylpiperidin-4-yl)-2,4-pentadienamide, a novel indole derivative optimized from bafilomycin, SB242784 exhibited inhibitory effect on bone resorption of chicken osteoclasts and human osteoclastoma in a low nanomolar range. Compared with the inhibition efficiency on V-ATPase complexes from other tissues including kidney, liver, spleen, stomach, brain, and/or endothelial cells, SB242784 showed a greater than 1000-fold selectivity for the osteoclast V-ATPase [228]. Administration of SB242748 for six months as a potential anti-osteoporosis therapy prevented bone loss in ovariectomized rats [228, 233].

**Table 5. Osteoclast-specific V-ATPase inhibitors.**

| Name              | Class of Derivatives | Targeting Sites | Selectivity | In vitro | In vivo | Other Effects |
|-------------------|----------------------|-----------------|-------------|----------|---------|---------------|
| SB242784          | indole derivative    | c, a, or V0     | high        | Inhibited retinoid-induced hypercalcemia in thyroparathyroidectomized rats Prevented bone loss in ovariectomized rats | Inhibited V-ATPase activity in chicken osteoclasts (IC50 of 29 nM), human osteoclastoma (IC50 of 22 nM) and human osteoclastic bone resorption (IC50 of 3.4 nM) | Anti-tumor activity in vitro and in vivo Inhibited V-ATPase-mediated intracellular acidification in osteoclasts with potent cytotoxicity |
| lejimalides       | 24-membered ring     | V0 domain       | low         | N/A      |         |               |
|                   | macrolides           |                 |             |          |         |               |
| FR167356          | benzamide            | unknown         | high selectivity in inhibiting osteoclast plasma membrane V-ATPase | Reduced retinoic acid-induced hypercalcemia in thyroparathyroidectomized rats Prevented bone loss in ovariectomized rats | Inhibited plasma membrane V-ATPase complex (IC50 of 190 nM) and lysosomal V-ATPase activity | Anti-tumor activity in vitro and in vivo Inhibited V-ATPase-mediated intracellular acidification in yeast cells Blocked macrophage and kidney V-ATPase activity |
| FR202126          | benzamide            | unknown         | low         | Reduced hypercalcemia induced by retinoic acid in thyroparathyroidectomized-OVX rats Prevented alveolar bone loss in experimental periodontitis in rats | Prevented bone resorption by murine osteoclasts (IC50 2.6-20 nM) | Unclear effects on other cell types expressing plasma membrane V-ATPases |
| FR177995          | benzamide            | unknown         | low         | Reduced bone loss in adjuvant-induced model of arthritis in rats; attenuated inflammation and articular cartilage damage | N/A | Non-specific inhibition of lysosomal and endosomal V-ATPase activity in dendritic cells |
| Diphyllin         | natural lignin       | unknown         | low         | N/A      |         | Anti-cancer, anti-inflammatory effects |
| KM91104           | Benzhydrazide        | a3-B2 interaction | medium      | N/A      |         | Phototoxicity, neurological problems, severe tendinitis, adverse immune activity, and renal failure |
| Enoxacin          | fluoroquinolone      | actin binding site on B2 | low         | N/A      |         | Anti-tumor agent Treatment of urinary tract infections and gonorrhea |
| Salisyllhalamide A | benzolactoneenamide  | V0 domain       | low         | Inhibited osteoclastic bone resorption and attenuated titanium particle-induced osteolysis in mice | | |
| (saliA), saliphenylhalamide (saliPhe) | | | | | | |

**Table 5. Osteoclast-specific V-ATPase inhibitors.**
FR167356, FR202126, and FR177995: This group of inhibitors exhibited a higher inhibitory effect on plasma membrane V-ATPase than on the lysosomal V-ATPase in osteoclasts and inhibited bone resorption in a dose-dependent manner. FR167356 also inhibited the V-ATPase complex of the kidney with similar efficacy [229].

**Diphylillin:** This compound has been shown to inhibit human osteoclastic bone resorption by inhibiting acid influx and lysosomal acidification compared with bafilomycin A1 [230].

**Salicylihalamide A (SaliA):** SaliA belongs to the benzolactoneenamide family of V-ATPase inhibitors and may act on the V₀ domain via a mechanism different from the classical macrolides such as bafilomycin and concanamycin [234]. A phenyl derivative of saliA, saliphenylhalamide (saliPhe), effectively inhibited osteoclastic bone resorption in a titanium particle-induced osteolysis mouse model [231].

**IEJLs:** These 24-membered ring macrolides were previously used in anti-tumor research and inhibited V-ATPase-mediated intracellular acidification in osteoclasts [232]. Other reports found that lejimalides A–D also inhibited lysosomal V-ATPase activity and induced S-phase arrest and apoptosis in MCF-7 cells and HeLa cells without inhibiting actin polymerization [235, 236].

**Artemisia capillaris:** The anti-osteoporotic activity of Artemisia capillaris has recently been reported. Its extracts diminished osteoclast differentiation and bone resorption, attenuated acidification, and reduced tumor necrosis factor receptor-associated factor 6 (TRAF6) expression and its association with V-ATPase [237].

**6.2 Targeting sites of V-ATPase inhibitors in anti-resorptives**

Subunit B2 of V-ATPase binds to F-actin with a profiling pocket-like structure. The inhibitor Enoxacin, a fluoroquinolone antibiotic, may target the interaction of F-actin and subunit B2 together with Binhib16 [216, 238, 239]. Some inhibitors target the interactions of V-ATPase subunits. The interaction between subunit a3 and B2 is crucial to the trafficking of ruffled border V-ATPase in activated osteoclasts that enables osteoclasts to keep acidified lacunae [136, 138, 213]. KM91104 could affect the interaction without changing cell viability or RANKL-mediated osteoclast differentiation [185, 219]. Luteolin, a naturally occurring flavonoid, could inhibit V-ATPase against a3-d2 interaction and reduce bone resorption without affecting the levels of these subunits and V-ATPase assembly [187-190]. The inhibition of bone resorption did not affect osteoclastic actin ring formation and cellular viability [191].

**6.3 Prospects of V-ATPase inhibitors as anti-resorptives**

We have an in-depth understanding of the complicated effects of V-ATPase complex in the bone system from the literature, human GWAS data, and mouse data from our own research. Defects in subunits a3 and d2 result in increased bone density. Many V-ATPase inhibitors are designed against these two subunits to reduce the osteoclast bone resorptive activity. Although subunit H stimulates the formation and resorptive activity of osteoclasts in vitro, its deficiency in vivo results in bone loss [86]. Thus, the traditional concepts based on the anti-resorptive V-ATPase inhibitors might not be adequate, and the directions of drug design need to be adjusted. The design of future anti-resorptive drugs should take a more comprehensive and multifaceted approach rather than focusing on a specific subunit of V-ATPase given the versatile functions of ATPases in osteoclasts as well as other cell types.

First, it is important to take into consideration the interactions among various subunits of V-ATPase that have direct physical interactions with a3-d2 and a3-B2. Meanwhile, the transcript levels of some V-ATPase subunits is changed with reduced levels of subunit H. However, the nature of these interactions between subunit H and those subunits is not clear and probably involves both direct and indirect contacts. Thus, a comprehensive understanding of the network of V-ATPase subunits will help us predict the net effect of V-ATPase in osteoclasts.

Second, the emphasis so far has been on the location of osteoclastic V-ATPases on ruffled borders and their function of extracellular acidification. In this review, we describe possible versatile locations and functions of intracellular V-ATPase via direct or non-direct involvements. As is evident by our Q-PCR analysis, the mRNA levels of most V-ATPase subunits are increased during osteoclastogenesis. The locations of these subunits in osteoclasts are not clear and may be either on the ruffled borders or in the intracellular components including endosomes, lysosomes, and ER. Most importantly, the functions of intracellular V-ATPases in osteoclasts may vary from pH regulation and endocytosis to protein recycling, as well as involvement in several signaling pathways such as Wnt, Notch, mTOR and GPCR pathways, which are essential for the development of bone and osteoclasts.

Third, the precise functions of most subunits of V-ATPase in osteoclasts are not well understood yet.
For example, besides influencing osteoclast function, subunit d2 affects bone mass by regulating osteoblasts. Subunit H targets the interaction between osteoclasts and osteoblasts through the TGF-β1 pathway. GEFOS analysis data imply that some subunits of V-ATPase are related to bone density without being present in osteoclasts.

In summary, in the future, direct or indirect functions of V-ATPases in osteoblasts as well as other cell types related to the bone system should be explored. Furthermore, the effects of V-ATPase inhibitors not only on osteoblasts but also on other cell types should be taken into consideration. It is of note that expressions of Tcirg1, Atp6vo1d2, and Atp6vo1b2 were recently detected in atherosclerotic lesions in mice, which might act against plaque calcification [240], providing an interesting perspective for the potential use of V-ATPase inhibitors in patients suffering from atherosclerosis and osteoporosis simultaneously. We believe that a new balance should be established between finding osteoclast-specific V-ATPase inhibitors or activators and considering their possible effects on other cells and tissues as well as their secondary effects on osteoclasts.

Related materials and methods

1. GEFOS and VEGAS analysis

GEFOS 2012 meta-analysis data including 32,961 subjects from 17 studies (http://www.gefos.org/?q=content/data-release) were used in our study [99]. SNPs in the genomic DNA of all V-ATPase subunits and the 50kb upstream and downstream region of certain genes were selected and analyzed with VEGAS methods [98]. The overall impacts of SNPs of a specific gene, as well as a single SNP, were compared.

2. In-house analysis

1627 Chinese volunteers were recruited from Midwestern Chinese Han adults living in Xi’an and Changsha cities. The analytical methods have been described previously [86, 100, 101]. Five groups were generated based on the Z-score; thresholds were -2, -1, 1, 2. All SNPs of V-ATPase subunits were selected and compared among groups (Table S2).

3. Osteoclasts culture and mRNA detection

At present, the locations and functions of V-ATPase subunits in osteoclasts have only been verified in a few subunits. To investigate whether more V-ATPase subunits are involved in osteoclastogenesis, osteoclasts were cultured, and Q-PCR was used to detect the mRNA levels of all V-ATPase subunits. The details are as follows: Bone marrow cells were separated from the femur and tibia of 6-week-old C57BL/6 mice and were treated with red blood cell lysis buffer (Solarbio, China) for 5 min. Subsequently, the cells were cultured in the presence of M-CSF (50ng/mL) (R&D, USA) for 24 hand then treated with M-CSF (50ng/mL) and RANKL (100ng/mL)(R&D, USA) for 4 or 7 days [86]. Total mRNA of cells at 0d, 4d, and 7d were extracted using the E.Z.N.A. total RNA kit (Omega, China). The PrimeScript™ RT Reagent Kit was used to synthesize cDNA (TaKaRa, Japan). Realtime PCR was performed to analyze the mRNA levels of V-ATPase subunits using SYBR® Premix Ex Tag™ (TaKaRa) and ABI 7500 real-time PCR system (Applied Biosystems) [241]. The primers of all subunits of V-ATPase are listed in Table S3.

4. RNA sequencing

Atp6vo1h knockout mice were generated using the CRISPR/Cas9 system. Osteoclasts were cultured as described above from wild-type and heterozygous mice (Atp6vo1h+/-). All animals were treated in accordance with the ethical guidelines of the School of Stomatology, the Fourth Military Medical University (Xi'an, China). Total mRNA of cells was extracted after induction with M-CSF and RANKL for 7 days [86]. High-quality RNA from each sample was combined into a single large pool to maximize the diversity of transcriptional units. The RNA library was constructed using Illumina’s TruSeq RNA Sample Preparation Kit (Illumina Inc, San Diego, CA, USA). The integrity of the RNA library was evaluated using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit). The amplified flowcell was used for paired-end sequencing on BGISEQ-500. After cleaning and quality checks, clean reads were generated. All coding genes of V-ATPase subunits were used to compare wild-type and heterozygous mice.

Supplementary Material

Supplementary tables. http://www.thno.org/v08p5379s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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