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Energy-Dependent Phosphate and Acid Transport for Bone Formation and Resorption

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ABSTRACT

Bone formation and resorption are mediated by an epithelial-like cell layer on bone. Formation or resorption requires active transport that depends on aerobic glycolysis, ATP, and acid transport. Metabolic activity of bone cells during matrix formation or removal is so high that the cells autolyze rapidly after cell death. Mineralization of bone matrix uses import of phosphate by sodium-phosphate cotransport, supported by the Na^+/K^+ ATPase. Glucose is the main energy source; ATP is exported to generate phosphate for hydroxyapatite in the bone matrix. Mechanism of export is not established, but phosphate is generated at least in part via phosphatase/pyrophosphatase activity including the tissue nonspecific alkaline phosphatase (TNAP) and ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2). Ca^{2+} is imported by paracellular transport. Protons, generated in producing hydroxyapatite, are exported by apical H^+/Cl^- exchangers ClC3 and ClC5, and basolateral Na^+/H^+ exchange. In bone resorption, ATP-dependent acid transport, the reverse of acid transport in bone formation, is essential. This uses the vacuolar-type H^+ATPase linked to Cl^- transport via a ClC family H^+/Cl^- exchanger, ClC7, and a Cl^- channel. Other transporters contributing include carbonic anhydrase and chloride-bicarbonate exchange to replace H^+ equivalents exported for bone resorption.

New and Noteworthy: This focused short review considers the relationship of oxidative phosphorylation to acid transport in bone formation and resorption, processes with very high metabolic activity for storage or removal of phosphate, calcium and acid equivalents.

1 | Introduction

Acid transport is required to support hydroxyapatite synthesis by, osteoblasts, and to mediate hydroxyapatite removal in bone repair or remodeling, by osteoclasts. We do an overview of major phosphate-producing and acid-producing transporters for bone production or resorption, with brief indication of context and supporting ion transporters. In subsequent sections, linkage of major cotransport support mechanisms, some in part hypothetical, are discussed in more detail.

Osteoblasts import phosphate and Ca^{2+}, and form hydroxyapatite mineral, which produces large amounts of acid:

$$6\,HPO_4^{2-} + 2\,H_2O + 10Ca^{2+} \leftrightarrow Ca_{10}(PO_4)_6(OH)_2 + 8\,H^+ \quad (1)$$

This requires support by major transport processes that are either directly ATP dependent or dependent on active transport secondarily linked to cellular energy metabolism. Active bone cells are highly metabolically active, and autolyze when isolated, so rapidly that investigators viewing sections of bone are not aware of the epithelioid osteoblast surface mediating transport [1].

Briefly, mineralized bone matrix production includes import of phosphate by sodium-phosphate cotransport by the neutral phosphate transporter-2 (NPT2) [2], supported by the Na^+/K^+

ATPase. Glucose and other intermediate substrates are imported to support this transport in osteoblasts; in bone formation phosphate from ATP is exported for hydroxyapatite synthesis. The mechanism is not fully established, but activity requires phosphatase/pyrophosphatase activity major mediators being the tissue-nonspecific alkaline phosphatase (here, abbreviated alkaline phosphatase) and the ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) [3, 4]. ATP export and dependency of bone formation on phosphate are documented in multiple contexts [5–7], but specific mechanisms of ATP transport are unclear. Ca^{2+} is imported by paracellular transport.

Protons generated in producing hydroxyapatite are exported by apical H^+/Cl^- exchange and basolateral Na^+/H^+ exchange [8]; these are in turn supported by additional Na^+/K^+ ATPase and $Na^+/K^+/Cl^-$ cotransport. Occurrence of cation transport has been reviewed [9]. Conversion of ATP to phosphate is essential: Free ATP or pyrophosphate inhibit mineralization [10].

Although known mainly for vesicular transport, H^+/Cl^- antiporters ClC3 and ClC5 occur at the bone apical surface. This was initially suspected from Cl^- dependency of H^+ transport in osteoblast membranes [11], and later confirmed using knockout mice [8].

ATP-dependent acid transport is also essential in bone resorption by osteoclasts. This is Equation 1 (above) operating in reverse. Important parallels between acid transport in bone formation and bone resorption include that the osteoclast vacuolar-type H^+ATPase is linked to Cl^- transport [12]; including surprising dependency on the ClC family H^+/Cl^- antiporter ClC7 [13], although at least one Cl^- channel also occurs at the acid-producing membrane [14]. Many other transporters contribute to H^+-ATPase activity. These include carbonic anhydrase and chloride-bicarbonate exchange that replace H^+ equivalents exported.

2 | High Turnover Bone Is Energy Intensive

2.1 | Direct Effects of Rapid Bone Turnover on Temperature

This is a fascinating but rarely discussed issue. Briefly, healing fractured bone has measurably increased joint temperature for over 10 weeks [15]. This interesting topic has not been studied in detail. We suggest that this reflects, in major part, the high metabolic activity of bone during degradation and synthesis of new bone. Without belaboring the point, it has long been recognized that arthritic joints are warm and respond to treatment, such as corticosteroids, with normalization of temperature [16]. One of the interesting consequences is that in regions with calorie-limited diet, skeletal growth and height are reduced in children; in Asian countries after World War 2, generations with more food are taller [17]. After 1980, in Japan the increase stopped [18].

2.2 | Glucose and Oxidative Phosphorylation In Bone

Considering the subject in detail, energy is required to synthesize hydroxyapatite. Includes an ATP to ADP step for each molecule of phosphate taken up by the osteoblast (six per mole of hydroxyapatite) and another ATP to ADP for each proton exported for new mineral, to supply Na^+/K^+ ATPase (Equation 1 and Figure 1). Similarly, a minimum of two moles of ATP must be converted to adenosine and Pi per mole of hydroxyapatite, and the function of acid uptake in osteoblasts is also indirectly energy dependent, likely via $Na^+/Cl^-/K^+$ transport, since proton uptake is mediated, at least in major part by outward chloride transport (Figure 1). All of the high-energy phosphate is almost certainly produced from oxidation of glucose [19]. This produces approximately 32 moles of ATP per mole of glucose [20]. Details of individual transport processes are discussed below. As in all cells, glucose uptake is via the Glut family of genes, SLC2.

3 | Bone Formation and Resorption Components and Mechanisms

3.1 | Bone Mineral Components in More Detail

Hydroxyapatite as diagrammed in equation 1 above, $Ca_{10}(PO_4)_6(OH)_2$, is an approximation of bone mineral that is useful in describing bone matrix. It is 95% or more of bone mineral [21]. There are important substitutions of ions including divalent cations, mainly magnesium on the order of 0.5% [21] and anions, mainly carbonate, generally in the 4% or less [22]. At these concentrations, magnesium and carbonate do not appear to compromise the strength of bone mineral. Since carbonate is ubiquitous in biological solutions at ~20 mM, its incorporation into hydroxyapatite is not surprising and will not further be discussed. Magnesium is a serum component at ~1 mM, and about twice that intracellularly where Mg^{2+} is essential for enzymes that use ATP. Magnesium is clearly important in bone health [23], but mechanisms including roles in organs other than bone also will not be discussed as outside of our focus. On the other hand, hypothetically, the minor substitution of Mg^{2+} for Ca^{2+} of ~0.5% in bone might be regarded as lack of specificity for calcium transport which occurs between osteoblasts as discussed below. Alternatively, phosphatase action on exported ATP is dependent on Mg^{2+} as with all ATP-dependent enzymes. With modern methods, many other ions are detectable; issues of other minor components are beyond the scope of this review.

4 | Phosphate Transport into Osteoblasts

4.1 | Na-Phosphate Cotransport

Uptake of phosphate in osteoblasts was studied in mouse calvarial osteoblasts [2], where it was shown to require the type 2 sodium–phosphate co-transporter (NPT2). The two genes SLC20A1/A2 both are highly expressed in human osteoblasts in our Affymetrix studies (Figure 1). The action of NPT2 in mouse calvarial osteoblasts required the PDZ-domain containing protein NHERF-1 [2]. NHERF-1, otherwise known as the Na^+/H^+ exchanger regulatory factor 1, is

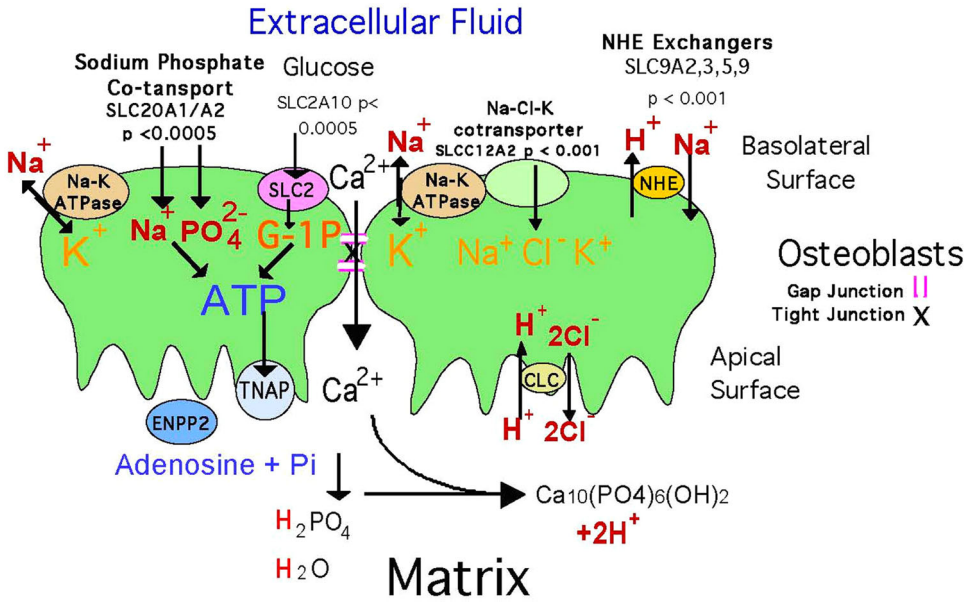

FIGURE 1 | Major metabolism-linked transporters in osteoblasts, emphasizing phosphate uptake and production of extracellular phosphate (left side) and novel ClC chloride-proton exchangers remove acid in the apical membranes (right side). For each major transporter illustrated, ion transport required for cellular neutrality, known to be present in gene screening data (see Data Availability) is illustrated. For brevity, this is discussed in the main text under sections labeled for the major transporters. Specifically, phosphate is imported into osteoblasts, at least in major part, by neutral phosphate transporters SLC20A1/A2 [2]. It produces ATP as an intermediate as very little free phosphate exists in osteoblasts. Phosphate for extracellular mineral is subsequently produced by the tissue-nonspecific alkaline phosphatase (TNAP) [4] and the recently described endonuclease-phosphatase extracellular phosphatase (ENPP2). Mechanism of transport of adenosine phosphates is controversial but there is no doubt as to the phosphate production for hydroxyapatite. Several mechanisms for phosphate transport are discussed in the section Phosphate Transport out of Osteoblasts. Stoichiometry is estimated [1], ~10 ATP are required per mole of hydroxyapatite. ClC chloride-proton exchange proteins are required for bone formation [8]. Regulation of ClC channel function in osteoblasts is not described, but is likely to be voltage dependent and to involve other proteins. Confirmation of ClC involvement used knockout mice [8], we separately showed that acid uptake is Cl^- dependent [42]. Gene names and data are from human osteoblasts analyzed twice each with $n = 12$ (Affymetrix), described in our published work [41] and available as described in Data Availability. All genes named are highly expressed in osteoblasts.

a scaffold protein interacting with several proteins that link membrane and cytoskeletal proteins, in this case shown by NHERF-1 knockout mice to be regulated, logically enough, by parathyroid hormone [2]. Sodium-phosphate transport is highly energetically favored since both outside to inside sodium and outside to inside (free) phosphate have large gradients. The NHERF-1 protein also regulates the obviously necessary Na^+/K^+-ATPase [24], also found, as expected, abundantly in human osteoblasts and mouse calvarial cells (Figure 1). The linkage to the ATPase is a major contributor to the energy dependency of bone formation.

5 | Phosphate Transport out of Osteoblasts

Detailed mechanisms [25, 26] have been proposed without a clear connection to mineralization being confirmed. The vesicular nucleotide transporter SLC17A9 [25] might traffic ATP and other nucleotides to vesicles for release to the bone forming space, but detailed description of such a mechanism is lacking beyond that SLC17A9 regulates osteoblast differentiation [25]. Mechanically stimulated ATP release [26] is interesting, although mechanistic detail are lacking, but could involve vesicular contents. Additionally, the plasma membrane protein ANKH regulates ATP transport [27]. The mechanism is unclear, and ANKH is not itself a transport protein.

Overall, the most promising mechanism is vesicular ATP transport. It is established that osteoblasts produce matrix vesicles that participate in early stages of mineral formation, although how ATP supports phosphate release and how it is exported in vesicles is unclear, and alternative hypotheses are promoted [28]. In related work, transport of Type I collagen is massive and active during matrix formation and mineralization. This collagen transport, definitely vesicular, is extensively reviewed with interesting data on collagen defects [29]. But: linkage to ATP in the vesicles is not studied. Collagen processing and vesicular delivery typically does not address the presence of ATP.

Other possibilities include direct transport through CX43 hemichannels [30]. This is attractive in that hemichannels would conduct ATP from the intracellular space to the extracellular space. There are many precedents for CX43 hemichannels in osteoblasts and osteocytes. On the other hand, CX43 deficiency is not fatal to osteoblasts or bone formation [31], despite extensive evidence of its importance in bone physiology [32]. Thus, ATP transport through CX43 hemichannels might occur, but it is not an exclusive or essential pathway. In mitochondria, ATP is trafficked by translocation generally of ADP imported and converted to ATP [5, 33]. There

is no evidence for ATP-specific mechanisms in secretion in the osteoblast's apical (bone forming) membrane.

A related matter on which there is very little data is that any release of ATP at the osteoblast's apical surface would require adenosine or nucleotide uptake into the osteoblast. Bone matrix does not contain ATP or adenosine in measurable quantities. The P2Y adenosine receptors are highly expressed in osteoblasts [34], suggesting the obvious hypothesis that phosphatases liberate phosphate in the extracellular space and that adenosine is simply recycled by receptor internalization.

6 | Calcium Transport into Bone is Paracellular

6.1 | TRPV Channel Proteins and Regulatory Claudins

The distinguished researcher Carol Gay and co-workers studied calcium transport through osteoblasts very carefully, and concluded that although osteoblasts, as expected, have sodium/calcium exchangers, their location and activity are not consistent with transcytosis of calcium for mineral formation [35]. Subsequently, calcium-selective TRPV5/TRPV6 channel proteins [36] associated with tight junctions were discovered. These and regulatory proteins, claudins, transmit calcium in a highly selective manner. This is important in a number of contexts including in the kidney [37]. TRPV5 is significantly expressed in human osteoblasts. For simplicity, it is not included in Figure 1.

7 | Acid Transport out of New Bone Matrix

7.1 | Acid Transport at the Osteoblast Basolateral Surface Occurs by Na^+/H^+ Exchange

This topic was explored In Vitro and In Vivo with definitive results pointing to extremely active Na^+/H^+ exchange [38]. It is particularly interesting to note that when sodium is replaced in osteoblast supernatants with N-methyl-D-Glucamine, 40 mM proprionate loading caused dramatic decrease in intracellular pH, on the order of two full pH units, which was reversible after 2 min by replacing supernatant sodium. Both this and the sodium phosphate uptake exchanger are regulated by the sodium hydrogen exchanger regulatory factor 1 [2]. Coordinated regulation of multiple transporters is efficient and appropriate for multiple energy intensive functions.

7.2 | H^+/Cl^- Antiporters ClC3 and ClC5

How osteoblasts remove the massive acid produced by bone formation has been difficult to approach and remains to some extent controversial. Possibilities include the proton conductance (outgoing) that is a component of the V-ATPase membrane subunit [39], although this is relevant only in acid transport in the osteoclast. We considered many possibilities, and with direct study of acid uptake in murine osteoblasts found this outward acid transport to be chloride dependent [11]. This was unexpected with literature on Cl^-/H^+ exchange mainly found in vacuolar chloride transport [40]. However, on review of overall osteoblast protein expression by Affymetrix analysis [41] we found apparently unreasonably high expression of ClC3 and ClC5 $2Cl^-/H^+$ exchangers and evaluated these in osteoblasts [11] and knockout mice for ClC3 or ClC5 [8]. This showed that either ClC3 or ClC5 when absent decreased bone mineralization, and that if both are absent this is fatal. Further, when ClC3 is absent ClC5 expression is greatly increased [42]. For interested readers, an Affymetrix analysis of osteoblast mRNA expression is provided; see "Data Availability"). The $2Cl^-/H^+$ mechanism also requires a large amount of chloride; our hypothetical model includes the highly expressed $Na^+/K^+/Cl^-$ cotransporter [41]. Unstudied additional questions include how excess chloride is removed from new bone matrix; this might include chloride channels such as CLIC4, which is highly expressed in human osteoblasts [41], not shown in Figure 1. Other possibilities include calcium-dependent chloride channels, which have not been studied.

8 | Transport of Sodium and Chloride for Cellular Neutrality

The osteoblast requires outward transport of Na^+ and Cl^-. Specifically, Na^+/PO_4^- cotransport and Na^+/H^+ exchange require elimination of a large amount of sodium, the role of the Na^+/K^+-ATPase [24] which is abundant in the osteoblast (Figure 1). This is a major component of the energy cost of bone production, as outlined above. Energy balance in chloride is the role of the K^+/Cl^-

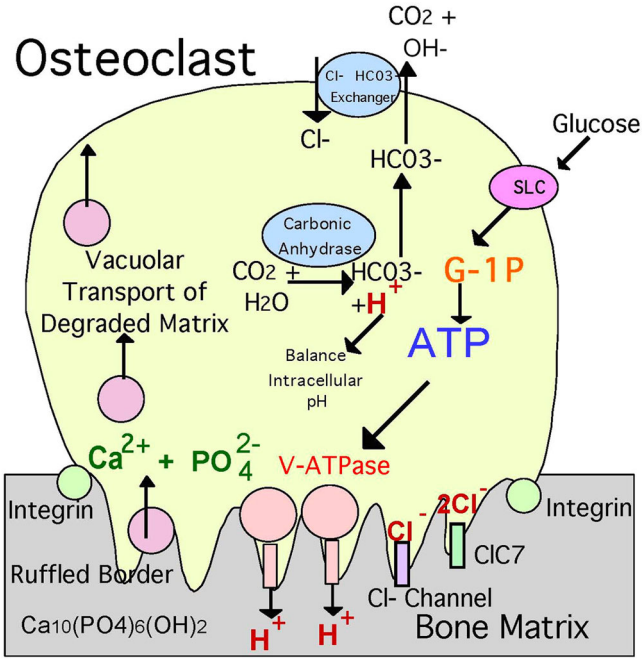

FIGURE 2 | Major metabolism-linked transporters in osteoclasts. As in transport for bone formation, massive production of ATP is essential. Electrogenic H+ transport is balanced by ClC7 chloride-proton exchange and a chloride channel to remove acid in the apical membranes [13, 14]. Details of counterions for the V-ATPase are unclear, but knockout of ClC7 causes a form of osteopetrosis. See Figure 1 for more detail on ClC exchangers. As in mineral deposition, roughly 10 ATP per molecule of HAP are needed. Products are removed by vacuolar translocation, e.g. [53]. Secretion of a massive amount of acid would alkalinize the osteoclast; this is managed by carbonic anhydrase and chloride-.

transporter and $Na^+/Cl^-/K^+$ cotransporter [43], both highly expressed in the osteoblast (Figure 1); for simplicity only the $Na^+/Cl^-/K^+$ is illustrated.

9 | Osteoclast Acid Transport

9.1 | Acid Transport to the Apical Surface: V-Type ATPase

It was surprising to find in 1989 that a V-Type ATPase is highly expressed at the osteoclast's apical membrane, and that this is essential for bone resorption [44]. Subsequent work showed surprising variation in subunits of the V-ATPase, particularly of the a3 subunit in different tissues [45]. There is a great deal of quality work on details, including targeting of the V-ATPase to the expanded (ruffled) membrane [46] (Figure 2). It is impractical to discuss all in a focused review, although it is worth noting that work has included differential expression in various organs beyond the osteoclast [47]. A number of specialized names for V-ATPases subunits have been developed. Those interested in assays of V-ATPase components in osteoclasts are advised to be cautious in selecting antibody or PCR targets.

9.2 | Osteoclast Attachment Limited by Specialized Integrins Binding to Bone Matrix

It was once believed that the osteoclast might function without a tight attachment to bone, but work in integrin-mediated binding in the 1990s, particularly integrin alpha$_v$beta$_3$ ($\alpha_v\beta_3$) [48] showed that osteoclasts, with typically large surface area and tight attachment, are capable of producing pH low enough to degrade bone essentially continuously (Figure 2). Integrin $\alpha_v\beta_3$ activates the non-receptor tyrosine kinase (NRTK) c-Src. Activation of the c-Src leads to a cascade that results in the polymerization of F-actin, which forms the actin-ring with podosomes. The podosomes directly attach to the bone matrix. M-CSF increases affinity of $\alpha_v\beta_3$ and activates the Rho family GTPase Rac in an integrin $\alpha_v\beta_3$-dependent manner [48].

9.3 | ClC7, Bicarbonate-Chloride Exchanger, and Other Chloride Channels

The V-Type ATPase is electrogenic, so that it cannot function without a charge-equalizing transporter. The surprising discovery that ClC7 is expressed in the osteoclast, where its deficiency causes a form of osteopetrosis, fits this role since it can function as Cl^-/H^+ exchanger [49]. The osteoclast has a number of chloride channels also important in function of the V-ATPase [50]. The bicarbonate-chloride exchanger at the plasma membrane of osteoclasts is important for regulating the cytoplasmic pH during bone resorption. The role of the additional chloride channels or putative chloride channels identified in osteoclasts, volume-regulated anion channels Chlor.62 and CLIC1 has not been established. In the osteoclast, additional processes supporting bone resorption are carbonic anhydrase for production of bicarbonate from CO_2 as a source of protons, and HCO_3^- exchange to eliminate the anion product. A cell exporting large quantities of acid would become alkaline, not compatible with

TABLE 1 | Key papers related to specific topics in bone mineral transport.

	Key references
General Bone Physiology	
1) The equation for hydroxyapatite formation	[1]
2) The effect of bone turnover on tissue temperature	[15]
3) Glucose and oxidative phosphorylation in bone	[19, 20]
Osteoblast Key Transport Processes	
1) Detailed components of bone including ion substitution	[20–22]
2) Basolateral Na-phosphate cotransport and linked transport proteins	[2, 24]
3) Apical phosphate transport out of osteoblasts	[25–29, 34]
4) Paracellular calcium transport into bone	[35–37]
5) Basolateral acid transport out of new bone -- Na^+/H^+ exchange	[2, 38]
6) Supporting highly expressed sodium transporter	[24]
7) Apical acid transport out of new bone -- chloride dependent	[8, 11, 42]
8) Supporting highly expressed chloride transporters	[41, 43]
Osteoclast Key Transport Processes	
1) H^+-ATPase at the apical surface and supporting data	[44–47]
2) Supporting chloride transporters	[49, 50]
3) A key attachment supporting integrin, alphavbeta3	[48]
4) Osteoclast reversible binding and osteomorphs	[54], [56]
5) Vesicular translocation of osteoclast resorption products	[53]
6) Basolateral regulation of cytoplasmic pH	[51, 52]

continued function. The osteoclast expresses large quantities of carbonic anhydrase producing intracellular protons and carbonate; the enzyme functions in multiple tissues and the phenotype is complex [51] (Figure 2). The carbonic anhydrase requires a mechanism to eliminate the carbonate base, which is a highly expressed chloride-bicarbonate exchanger [52] (Figure 2).

10 | Translocation of Osteoclast Resorption Products

10.1 | Vesicular Uptake, Transcytosis

How resorption products are released was the subject of speculation for many years. The essential answer is vesicular transcytosis [53]. Regulation of vesicular trafficking processes is mediated by Rab proteins in the family of small GTPases. Rabs cycle between an inactive, GDP-bound state and an active, GTP-bound state. In their active state, Rab proteins interact with numerous effector molecules to control multiple aspects of vesicle trafficking, including budding, transport and fusion [54].

Osteoclasts are, surprisingly, capable of detachment and separation, in which large polykaryons separated into smaller daughter cells, called osteomorphs, capable of further differentiation [55]. They not only migrate away from the parent polykaryon, but they also fuse with neighboring osteoclasts and in some cases with each other. Thus, osteomorphs are "recycled" into larger polykaryons. These findings were made possible by new labeling techniques in living cells. Thus, the energy-dependent resorption activity is not a one-way street, but reversible, at least in part.

11 | Summary

We summarize key elements of transporters involved in bone synthesis and degradation as summarized in Figures 1 and 2. For the convenience of readers, key papers on specific transport processes are summarized in Table 1. It is important to consider that the process of bone formation and degradation is quite complex, and thus, avoiding false impressions is important. A major such issue is that while it is well known that glycolysis is key to energy production in the osteoblasts [52], it is important to consider that at any given time, typically 20% of osteoblasts are active, as shown by labeling bone formation with calcein or acridine orange in mice, while 80% are relatively metabolically inactive "lining" cells, illustrated in control cells in [11] and numerous other studies. To avoid unnecessary complexity, we do not review the variable energy metabolism of osteoblasts. Variability in bone resorption pathways is also important, with recent very interesting work on osteoclast reversibility of differentiation and activity [54] of evolving interest and importance.

Author Contributions

Irina Tourkova, Deborah Nelson, Paul Schlesinger, and Harry Blair: Conceptualization, writing; review and editing. References to work reviewed includes studies published by all authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The Affymetrix study of expression of proteins in human osteoblasts is available at http://d-scholarship.pitt.edu/45759/. This database is an Affymetrix assay of human osteoblasts.

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