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Osteoclasts Provide Coupling Signals to Osteoblast Lineage Cells Through Multiple Mechanisms

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Abstract

Bone remodeling is essential for the repair and replacement of damaged and old bone. The major principle underlying this process is that osteoclast-mediated resorption of a quantum of bone is followed by osteoblast precursor recruitment; these cells differentiate to matrix-producing osteoblasts, which form new bone to replace what was resorbed. Evidence from osteopetrotic syndromes indicate that osteoclasts not only resorb bone, but also provide signals to promote bone formation. Osteoclasts act upon osteoblast lineage cells throughout their differentiation by facilitating growth factor release from resorbed matrix, producing secreted proteins and microvesicles, and expressing membrane-bound factors. These multiple mechanisms mediate the coupling of bone formation to resorption in remodeling. Additional interactions of osteoclasts with osteoblast lineage cells, including interactions with canopy and reversal cells, are required to achieve coordination between bone formation and resorption during bone remodeling.

INTRODUCTION

Long-standing views that skeletal structure and function are regulated by circulating hormones have been superseded by overwhelming evidence of control by local factors within the bone itself. In particular, communication pathways coordinating skeletal renewal throughout life are now recognized as crucial for determining bone mass. Over recent years, many such pathways have been defined. This review focuses on describing the different classes of pathways by which osteoclasts (bone resorbing cells) direct and coordinate the actions of osteoblasts (bone forming cells). These include the production of coupling factors as (*a*) proteins released from the resorbed bone, (*b*) proteins secreted by osteoclasts, (*c*) membrane-bound proteins on the osteoclast surface, and (*d*) exosome-associated proteins and miRNAs released by osteoclasts. The process by which osteoblast activity is coupled to osteoclasts (coupling) also relies on actions that modify the local cellular geography, including forming a resorption pit, lifting an overarching canopy, and cellular interactions within the reversal phase that occurs between bone resorption and formation phases. By understanding these mechanisms, we can gain a better understanding of skeletal biology, identify targeted approaches by which bone mass can be modified for therapeutic benefit in conditions such as osteoporosis, and develop new concepts to help understand cell–cell communication mechanisms.

BONE MODELING, REMODELING, AND THE COUPLING PROCESS

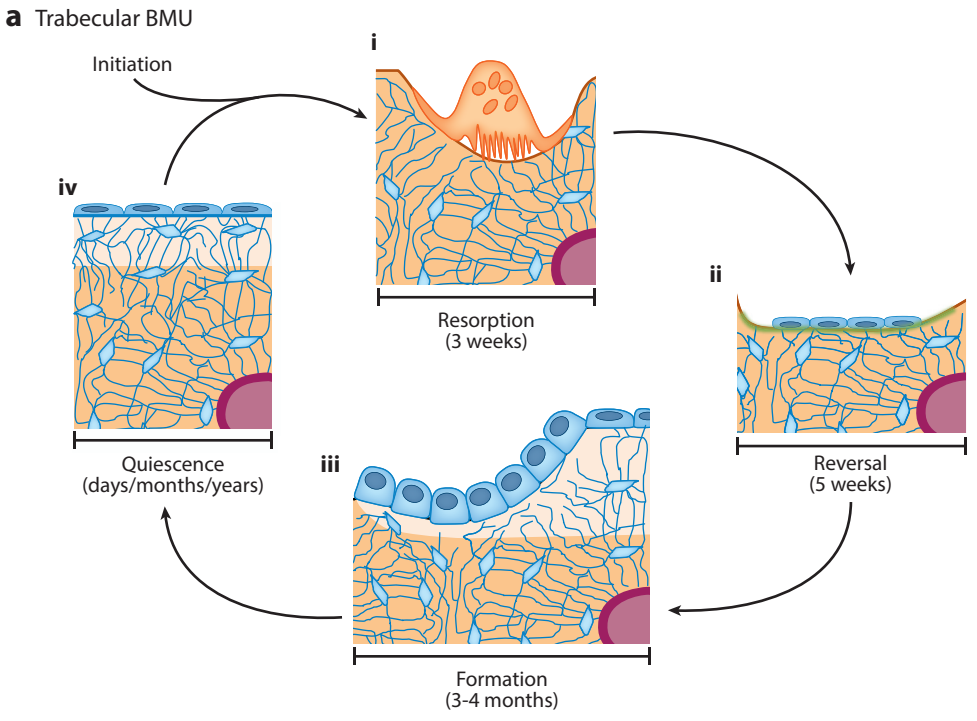
Skeletal structure is determined by two coordinated processes: modeling and remodeling. In essence, their difference lies in when and where cells that resorb bone (osteoclasts) and cells that form bone (osteoblasts) act relative to each other. In modeling, bone-forming osteoblasts and bone-resorbing osteoclasts act at separate locations (i.e., on different surfaces) but often at the same time. In contrast, during remodeling, osteoblasts and osteoclasts act on the same bone surface, but in sequence, with osteoclast activity preceding osteoblast activity. Modeling and remodeling may also differ in their ultimate effects on the skeleton: modeling changes bone shape, whereas remodeling can renew the structure while maintaining the same shape.

Modeling modifies skeletal shape. From the start of bone development to the end of the second decade when the longitudinal growth is completed, modeling determines bone construction (1) and shape (2, 3). In modeling, bone formation occurs independently (i.e., without prior bone resorption), as does bone resorption (without subsequent bone formation). In this way bone is formed at sites of greatest mechanical load and removed where it is not required. For example, bone formed and deposited on the outer surface widens the lengthening bone; at the same time, bone is resorbed at the endosteal surface to enlarge the cavity housing the bone marrow. Although these actions must be coordinated to result in the predictable sizes and shapes of individual bones, these likely are programmed initially by developmental and mechanical cues and during puberty by systemic actions of sex-steroid hormones; the precise mechanisms of modeling control remain largely undefined.

Bone remodeling is the process by which the adult skeleton is renewed by removing and replacing damaged or old bone. The concept was introduced by Frost in examining multiple sections through trabecular bone from the normal adult rib (4). Its role in bone rejuvenation remains the prevailing view, and there have been many efforts to explain it in cellular and molecular terms (2, 5–7). The sequence of remodeling is as follows (**Figure 1*a***). Osteoclast formation is initiated, and a tiny amount of bone is removed. This is then replaced by new bone formed by osteoblasts. This new bone undergoes primary, then a much slower secondary, mineralization. These sites of remodeling activity (1, 4), known now as basic multicellular units (BMUs) (2), are distributed asynchronously throughout the skeleton. At any one time, BMUs throughout the skeleton exist

at many different stages of the remodeling cycle—some resorbing, some forming, and some in reversal phase.

Structural differences can readily be seen between trabecular and cortical remodeling, but the sequence and principles operating are the same (**Figure 1**). Whereas in trabecular bone the BMU is located on a more or less flat bone surface and resorbed, lacunae can readily be seen in human



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Basic orientation of the basic multicellular unit (BMU) in trabecular and cortical bone. (*a*) In trabecular bone the BMU exists on the bone surface, and the cells on that surface change over time. (*i*) After initiation, osteoclasts resorb bone. This is followed by a reversal phase (*ii*) when osteoblast lineage cells line the bone surface to prepare it for bone formation. Osteoblasts then attach to the bone surface (*iii*) and form new matrix until the pit left by the osteoclast is refilled. During the quiescence phase (*iv*) that follows, the matrix continues to accrue mineral. The approximate duration of each stage reflects data obtained from human BMUs. It is not always possible to see all the stages at the same time. (*b*) BMUs within Haversian cortical bone exist within an osteon, and it is possible to see multiple stages of the remodeling cycle along a single cutting cone. (*i*) Osteoclasts tunnel by resorption into the bone, with a central blood vessel providing precursors. (*ii*) Osteoblast lineage cells line the bone surface during the reversal phase. (*iii*) This is followed by differentiated, matrix-producing osteoblasts that fill the central canal with new osteoid. This osteoid is gradually mineralized, an activity that continues during the quiescence phase (*iv*). A full description of the cell types and coupling mechanisms that may exist is depicted in **Figure 3**.

bone, and in Haversian cortical bone the BMU occurs within a pyramidal-shaped cutting zone; this is led by osteoclasts tunneling through bone, followed by differentiating osteoblasts (7–10). In both cases a reversal phase functions between the resorption and formation phases (11).

Because remodeling takes place in different parts of the skeleton at different times, locally generated and regulated factors must be important in ensuring appropriate communication among the participating cells. The location and extent of resorption by osteoclasts need to be tightly controlled, and so too do osteoblast differentiation (**Figure 2**) and the extent of bone formation within BMUs. The communication mechanism by which bone formation follows bone resorption is known as coupling, a term relating to the mechanism by which a train carriage follows the engine. Osteoclasts are the engine driving remodeling and, through coupling, osteoblasts are the cellular carriage that follows.

To maintain bone mass, the amount of bone removed in the initial resorptive stage at the BMU must be equaled by the amount replaced. If bone resorption and formation are not balanced, bone mass is not maintained. If bone formation is less than resorption, bone loss occurs (catabolic); conversely, if bone formation is more than resorption, bone mass increases (anabolic). Such a lack of balance does not mean osteoclasts and osteoblasts are uncoupled. This term should not be used in this circumstance. Bone formation still follows resorption at the BMU; they are still coupled, but the activities are unbalanced. The situations in which bone formation and resorption might be uncoupled are if bone formation were blocked entirely so that osteoblasts do not follow osteoclasts at the BMU. This can occur, for example, with stem cell mobilization therapies (12) and has been observed with glucocorticoid treatment in sheep (13).

This review focuses on the many mechanisms implicated in the coupling process and how each contributes to the sequence of remodeling and to its balance. It is important to understand that osteoclast activity does not always occur in BMUs and is not always followed by bone formation. Examples of these other osteoclast actions in bone modeling include site-specific resorption of the primary and secondary ossification centers during bone development to form the medullary cavity (14) and on the periosteal surface to reduce the metaphyseal width (15). Other examples are pathological bone resorption in inflammatory bone disease and the skeletal complications of cancer. While they may have some mechanisms and factors in common with coupling, they are not discussed in this article.

Understanding the multiple mechanisms by which osteoclasts communicate with osteoblasts during bone remodeling is clinically important because bone mass in the adult skeleton depends on bone remodeling balance. By identifying coupling mechanisms, we will gain an improved understanding of current osteoporosis therapies and may be able to identify improved methods. One key example is the therapeutic limitation imposed by the coupling process: anti-resorptive

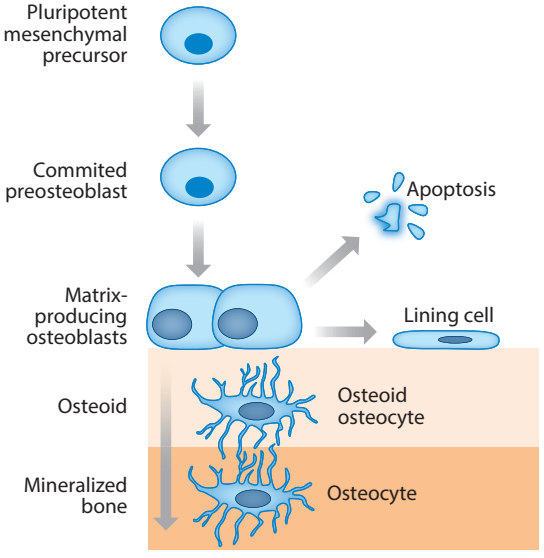


Figure 2

Stages of osteoblast lineage differentiation. Osteoblast differentiation is a slow process, and osteoclast-produced coupling factors may act upon any of these stages of differentiation to regulate bone formation. Osteoblasts are derived from pluripotent mesenchymal precursors, which become committed preosteoblasts following expression of *Osterix* and *Runx2*. They are attracted to the bone surface, where they attach and continue to differentiate into matrix-producing osteoblasts. Some of these cells embed within the osteoid matrix they produce and further differentiate into osteoid osteocytes, which, as the matrix becomes more mineralized, continue to differentiate and remain in residence as fully mature osteocytes. Those osteoblasts that do not differentiate into osteocytes undergo apoptosis or remain on the bone surface as lining cells. The differentiation process from mesenchymal precursor through to fully differentiated osteocyte takes approximately 3 weeks in vitro.

therapies, by inhibiting osteoclast formation or activity, also inhibit release of osteoclast- or matrix-derived coupling factors. This means they also inhibit osteoblast differentiation, and therefore cannot promote bone formation (16, 17). It is also a clinical challenge to increase bone mass by stimulating bone formation while simultaneously inhibiting bone resorption. This topic has been explored in detail very recently (17) and will not be discussed extensively here.

FACTORS RELEASED BY OSTEOCLAST-MEDIATED RESORPTION OF THE BONE MATRIX

The first proposed coupling mechanism was the release during resorption of growth factors stored in large amounts in the bone matrix (18). Transforming growth factor-β (TGF-β) and insulin-like growth factor-1 (IGF-1), factors known from cell culture studies to stimulate osteoblast differentiation, were revealed to be released and activated by the acid pH generated by osteoclasts during bone resorption (18). The hypothesis that osteoclasts released them locally to stimulate osteoblast precursors to differentiate in the local environment gained favor, although it was difficult to understand how the amounts of growth factor released could be tightly controlled. More recently, this hypothesis was refined by findings in genetically manipulated mice. TGF-β released during bone resorption acted as a signal inducing bone mesenchymal stem cell (osteoblast precursor) migration to resorption sites, thus making them available within the BMU for differentiation into bone-forming osteoblasts (19). Because these precursors require approximately a week to form

matrix-producing osteoblasts, controls to achieve balanced remodeling could be exerted at the later stages of their differentiation. This could be achieved through resorption-derived IGF-1, which was found to promote osteoblast differentiation by favoring recruitment of mesenchymal stem cells (MSCs) by activating mammalian target of rapamycin (mTOR) (19, 20). A further possible growth factor contributing to the coupling mechanism is homodimeric platelet-derived growth factor composed of two B units (PDGF-BB). PDGF-BB is also released from matrix and derived from both osteoblasts and osteoclasts (21). The ability of PDGF-BB to induce blood vessel formation may also provide progenitor cells for later differentiation into osteoblasts and bone formation (22).

Such mechanisms of initial precursor recruitment by released factors provide an attractive explanation for the concept that in remodeling, osteoblasts are recruited from a stem cell pool and need to be attracted to remodeling sites for differentiation (reviewed in 23). Because osteoclasts and matrix-producing osteoblasts do not reside within the BMU at the same time (see **Figure 1**), these mechanisms would also help to explain how osteoclasts can overcome the time delay of some weeks between the completion of bone resorption and the commencement of bone formation (5). Thus, factors released by osteoclasts during bone resorption are most likely to act to promote recruitment and migration of early osteoblast precursors to the bone surface (**Figure 3*b***), with their differentiation and bone formation levels controlled by later processes in the remodeling cycle.

OSTEOCLASTS SECRETE COUPLING FACTORS INDEPENDENT OF RESORPTIVE ACTIVITY

The concept that osteoclasts might also produce and secrete coupling factors, independent of their resorptive activity, arose from work in mice with elevated bone resorption due to elevated STAT signaling downstream of the gp130 cytokine receptor subunit (24). Those mice had elevated bone resorption and bone formation, but when they were crossed with interleukin (IL)-6-null mice, resorption remained elevated, but formation was suppressed (24). This suggested that resorption alone was insufficient to promote coupled bone formation and that the coupling pathway is IL-6 dependent. In addition, we suggested that coupling factors may mediate at least part of the action by which intermittently injected parathyroid hormone (PTH) stimulates bone formation within the BMU, a therapeutic approach used to increase bone mass in osteoporosis (25). Considering that early data showed that PTH had a rapid effect on osteoclasts (26) and that PTH anabolic action depended on the presence of osteoclasts (27, 28), we suggested the osteoclasts thus stimulated bone formation to result in an anabolic action (29).

Osteoclast-derived coupling factor release does not appear to depend on the resorptive activity of osteoclasts. This conclusion comes from the syndrome of osteopetrosis, high bone mass due to defective bone resorption. Osteopetrosis can result from one of two principal defects: failed osteoclast formation (osteoclast poor) or impaired osteoclast activity (osteoclast rich). Comparing these two forms of osteopetrosis led to the insight that coupling factor release does not depend on bone matrix resorption.

In osteoclast-poor osteopetrosis, such as in the rare individual human subjects with lost RANKL/RANK signaling (30, 31), or mice with genetic *c-fos* ablation (32), not only is bone resorption disrupted, but bone formation is also greatly reduced. This confirms that osteoclasts send a signal to osteoblasts to induce bone formation. That could still be explained by their release of the matrix-bound growth factors described above. However, in osteoclast-rich osteopetrosis, where bone resorption was impaired but osteoclasts were present, bone formation was normal, or even increased, rather than being reduced. These include mutations in chloride channel-7 (*ClCN-7*) (33) or the osteoclastic V-ATPase subunit A3 (also called *TCIRG1*) (34, 35), the

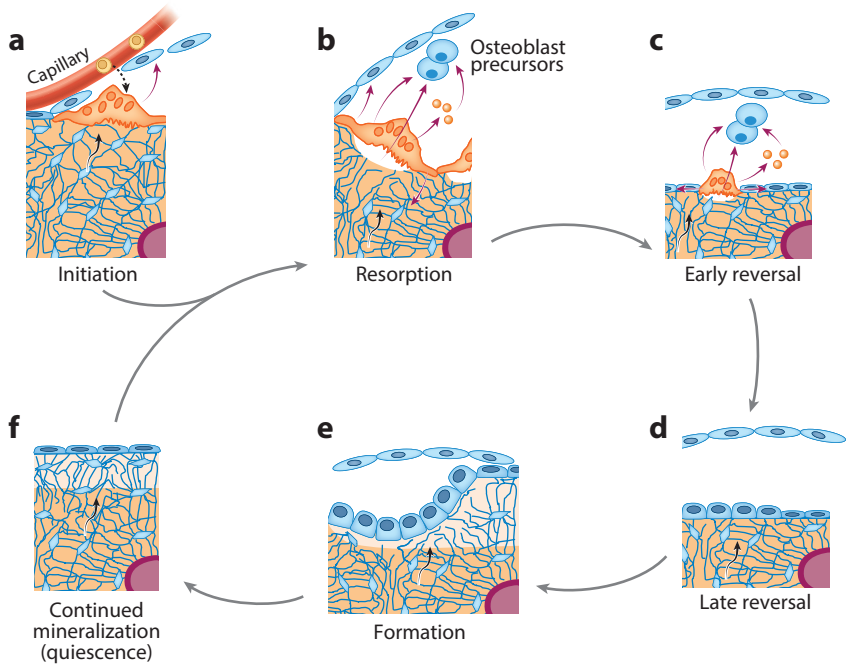


Figure 3

An updated model of remodeling with osteoclast-directed coupling signals (*purple arrows*) and osteocyte-derived signals (*black arrows*) highlighted at each stage. (*a*) During initiation of remodeling, which may result from an osteocyte-derived signal, lining cells lift to form a canopy, allowing osteoclast precursors entry to the basic multicellular unit through the bloodstream. It is possible for osteoclasts to signal to osteoblast lineage cells that form the canopy. (*b*) During bone resorption, osteoclasts may still interact with osteoblast lineage cells in the canopy. In addition, they secrete osteoclast-derived coupling factors, release products from the resorbed bone matrix, and release extracellular vesicles. All of these are likely to influence osteoblast precursors within the bone remodeling compartment enclosed by the canopy. They may also signal to osteocytes. In addition, osteocytes sense the increased strain on bone due to the reduced bone mass. (*c*) During the early reversal phase, small, less active osteoclasts remaining on the bone surface continue to release secreted factors, matrix-released factors, and extracellular vesicles, and they may make direct contact allowing contact-dependent signaling to osteoblast-lineage reversal cells on the bone surface. Osteocytes would continue to sense strain and send signals to osteoblast precursors. (*d*) During the late reversal phase, the number of osteoblast lineage cells increases, reaching a critical mass that promotes their bone-forming activity. Osteocytes continue to sense the mechanical strain and signal. Osteoclasts are not present and no longer exert direct control on the osteoblasts at this or subsequent stages. (*e*) During the bone formation phase osteoblasts deposit osteoid, which becomes gradually mineralized through rapid (primary) and slower (secondary) mineralization. Osteoblasts would sense the filling of the pit, and osteocytes may send signals when mechanical strain is reduced to stop bone formation. (*f*) In quiescence, flattened osteoblast-lineage-lining cells remain on the bone surface. When the bone surface is quiescent, mineralization continues until the bone is fully mineralized; this may be controlled by activity of osteocytes.

osteoclast motility molecules c-*src* (36) and *Pyk2* (37), and the matrix-dissolving enzyme cathepsin K (38). Even when osteopetrosis was induced in adult mice by transplanting hematopoietic precursor cells deficient in the osteoclastic V-ATPase subunit a3 (mouse gene name *Tcirg1*) (39), there was a significant reduction in resorption with no reduction in osteoclast numbers nor any reduction in bone formation in the recipient mice. Such effects have also been noted in humans with osteoclast-rich osteopetrosis; bone formation in these patients is normal or even increased

(40). This has been linked directly to increased numbers of nonresorbing osteoclasts by a study showing a direct correlation between the number of osteoclasts and the number of bone-forming osteoblasts (40). This indicated that osteoclasts do not need to resorb bone to release the coupling factors necessary to promote bone formation.

The ability of osteoclasts to stimulate osteoblasts independent of their resorptive activity was upheld by in vitro work in which cultured osteoblast-like cells could be stimulated to form mineralized nodules by conditioned medium from either osteoclasts cultured on plastic (therefore not resorbing bone) (41, 42) or nonresorbing osteoclasts generated from a patient with osteoclast-rich osteopetrosis due to a mutation in *TCIRG1* (43). A further in vivo study compared osteopetrosis induced by transplanting irradiated normal mice with osteoclast precursors from *oc/oc* mice (osteoclast-rich osteopetrosis) or with RANK-deficient (osteoclast-poor) cells (44). The increase in bone volume was larger with the *oc/oc* cell transplantation, despite a similar reduction in bone resorption, suggesting that the nonfunctional osteoclasts retained their ability to support bone formation also in vivo.

Having established that osteoclasts could synthesize and secrete coupling factors, the next challenge has been to identify them. Many osteoclast-secreted coupling factors have now been posited, most based on in vitro studies (shown in **Table 1**). Within the bone microenvironment, all these proposed coupling factors have multiple actions, and most are produced by multiple cellular sources. We discuss three of the earliest coupling factors identified as examples.

One of the first factors identified in in vivo studies was the gp130-signaling cytokine, cardiotrophin-1 (CT-1). In mice with global CT-1 deletion, although osteoclast numbers are high, their resorptive activity is low, and so too is bone formation, indicating reduced coupling factor production (45). CT-1 was detected in osteoclasts in situ by immunohistochemistry and shown to stimulate osteoblast differentiation in vitro and bone formation in vivo (45). Although the cellular source of CT-1 within bone seems to be restricted to osteoclasts, CT-1 stimulates bone formation through multiple mechanisms. These include action on early precursors to direct their differentiation to osteoblasts at the expense of adipogenesis (45) and action on osteocytes to suppress sclerostin production (46). CT-1 also stimulates osteoclast formation by promoting RANKL expression in the osteoblast lineage (47). CT-1 therefore has multiple effects, including coupling factor activity.

Two approaches have been used to identify coupling factors released by osteoclasts in vitro. One is to identify those factors produced by osteoclasts when precursors are stimulated with RANKL to induce osteoclast formation in vitro, and the other is to assess osteoclast-conditioned medium. Another early osteoclast-derived coupling factor [sphingosine-1-phosphate (S1P)] was found using both approaches (42, 48). S1P can have inhibitory or stimulatory effects on osteoblasts depending on differentiation stage and precursor source, such as human MSCs, immortalized MSCs, and mouse calvarial osteoblasts (42, 48). In vivo data suggesting a coupling role for osteoclast-derived S1P came from work in which osteoclast function was disrupted by deletion of the resorptive enzyme cathepsin K. This resulted in impaired resorption but retained coupling factor activity since osteoclast numbers and bone formation were maintained (49). The mutated osteoclasts from these mice had a greater capability to promote osteoblast differentiation in coculture, and this effect was inhibited by an S1P receptor antagonist. Although this suggests a role for S1P in the coupling process in the BMU, it needs to be explored further and put into the context of many other actions of S1P (reviewed in 50). S1P is expressed by multiple cells in the BMU, including the vasculature, where it acts on its receptor in osteoclast precursors to stimulate their recruitment in vitro (51). Paradoxically, S1P also suppresses bone resorption: In vivo and in vitro studies showed that S1P can limit osteoclast precursor chemotaxis and migration, essentially resulting in their increased recirculation from bone to blood (52). Furthermore, S1P receptor 1 (S1PR1) knockout

Table 1 A summary of osteoclast-derived coupling factors, their other potentially relevant cellular sources, and their other possible influences near or in the basic multicellular unit (BMU)[a]

Factor	Mode of action from osteoclasts	Other potentially relevant sources	Influences on osteoblast differentiation and bone formation	Other potential influences in remodeling
IGF-1, IGF-2	Matrix derived (103)	Osteoblasts (104) Macrophages (105)	Stimulates osteoblast progenitor expansion (20)	Stimulates osteoclastogenesis (106)
PDGF-BB	Matrix derived (103) Secreted (107)	Osteoblasts (108) Endothelial cells (109) Osteoclasts (110)	Promotes osteoblast precursor repli-cation (111), migration (112), and differentiation (111) Stimulates bone formation in vivo (113)	Stimulates osteoclast precursor recruitment (111)
BMP2	Matrix derived (114) Secreted (115)	Osteoblasts (116) Macrophages (117)	Stimulates osteoblast progenitor expansion, migration (118), and differentiation (119)	Stimulates osteoclast activity (120)
TGF-β	Matrix derived (114)	Osteoblasts (121) T lymphocytes (122) Macrophages (123)	Stimulates osteoblast progenitor expansion (121, 124), migration, and differentiation (19) Stimulates bone formation (organ culture) (124)	Acts on osteoclast precursors to stimulate osteoclastogenesis (125) Stimulates sclerostin expression by osteocytes (126)
CT-1	Secreted (45)	None	Stimulates bone formation in vivo (45) Stimulates osteoblast commitment (45) Suppresses sclerostin expression (45) Null mice have low bone formation (45)	Stimulates osteoclastogenesis (47) Bone resorption is low in null mice (45)
BMP6	Secreted (115)	Mesenchymal and hematopoietic stem cells (127)	Stimulates osteoblast differentiation (127)	Stimulates osteoclastogenesis from human marrow cells (128)
Wnt10b	Secreted (42)	T cells (129)	Stimulates osteoblast differentiation in vivo (130)	Stimulates osteoclast activity in vivo (130)
S1P	Production catalyzed by secreted sphingosine-1-kinase (42)	Vasculature (131) Red blood cells (132)	Promotes osteoblast precursor recruitment (42) Promotes osteoblast migration and survival (42, 48)	Stimulates osteoclast recruitment (131) and precursor chemotaxis (52) Inhibits osteoclastogenesis (48) Inhibition in vivo reduces osteoclast formation (53)
CTHRC1	Secreted (54)	Mesenchymal cells, osteoblasts (55) Osteocytes, circulates (56)	Stimulates osteoblast differentiation and bone formation in vivo (54, 55)	Inhibits osteoclast formation and activity (133)

(*Continued*)

Table 1 (*Continued*)

Factor	Mode of action from osteoclasts	Other potentially relevant sources	Influences on osteoblast differentiation and bone formation	Other potential influences in remodeling
Complement 3a	Secreted (134)	Circulating (50 ng/mL in human serum) (135)	Stimulates osteoblast differentiation (134)	Osteoclast recruitment (136)
Oncostatin M	Secreted (137)	Macrophages (138) Osteoblasts (46) Osteocytes (46) T lymphocytes (139)	Promotes osteoblast commitment (46) Stimulates bone formation in vivo (46)	Synergizes with BMP2 (137) Stimulates osteoclastogenesis (140)
CXCL16	Secreted (141)	Vascular smooth muscle cells (142) Macrophages (143)	May stimulate osteoblast precursor migration (141)	None
Leukemia inhibitory factor	Secreted (141)	Mesenchymal stem cells (144)	Stimulates bone formation in vivo (145) Stimulates osteoblast precursor expansion (146), differentiation (147) Inhibits sclerostin expression (46) Gene deletion leads to low bone formation in remodeling (147)	Stimulates osteoclastogenesis (148) Inhibits marrow adipogenesis (147) Inhibits vascularization (147)
SLIT3	Secreted (149)	Osteoblasts (150)	Stimulates osteoblast migration (149)	Inhibits osteoclastogenesis (149)
Semaphorin 4D	Membrane bound (65)	T lymphocytes (also soluble forms) (68)	Inhibits bone formation, and gene deletion increases bone formation (65)	Stimulates osteoclastogenesis (151)
EphrinB2	Membrane bound (61)	Osteoblasts (61) Osteocytes (60)	Promotes osteoblast differentiation (61) Suppresses osteoblast apoptosis (59) Promotes late stage osteoblast differentiation in vivo (152)	Inhibits osteoclastogenesis (61) Inhibits RANKL production by osteoblasts (59)
miR-214-3p	Exocytosed (78)	Monocytes	Suppresses osteoblast differentiation (78)	None
RANKL/RANK reverse signal	RANK: membrane bound (70) and exocytosed (75)	RANKL: osteoblast precursors, osteocytes (153)	Promotes bone formation (70, 75)	None

[a]The listed influences are in vitro, unless otherwise indicated.

Abbreviations: BMP, bone morphogenetic protein; CT-1, cardiotrophin-1; CTHRC1, collagen triple helix repeat containing 1; IGF, insulin-like growth factor; PDGF-BB, platelet-derived growth factor composed of two B units; S1P, sphingosine-1-phosphate; SLIT3, slit homolog 3; TGF-β, transforming growth factor-β.

in mice led to bone loss due to enhanced osteoclast attachment to bone surfaces, and treatment with FTY720, a drug agonist of S1PR1 and other S1P receptors, prevented resorption-induced bone loss in ovariectomized mice (52). Most recently it has been shown that increasing endogenous S1P, by either genetic or pharmacological means, increased bone mass and strength in mice (53). However, this was not caused by increased bone formation: Although mineral appositional rate was increased, implying more rapid primary bone mineralization, osteoid surface was low, which suggested impaired collagen production. Instead, the positive effect of increased S1P on bone mass seems to have been mostly caused by antiresorptive effects. Concentrations of the RANKL decoy receptor osteoprotegerin were increased, and osteoclast numbers were lowered. At this stage it seems that, even though S1P attracted much interest as an osteoclast-derived coupling factor that promotes bone formation, its primary role in vivo is rather to inhibit osteoclast formation.

A third example is collagen triple helix repeat containing 1 (CTHRC1), which has also been reported to be produced by actively resorbing osteoclasts and to stimulate osteoblast differentiation in vitro and bone formation in vivo (54). The identity of the key CTHRC1-producing cell is argued and needs to be established if CTHRC1 is to have a coupling role. Whereas it was suggested, using in situ hybridization, that CTHRC1 was produced by osteoclasts and not by osteoblasts (54), others found it was produced by the osteoblast lineage, including mesenchymal precursors (55), osteoblasts, and osteocytes (56). These differences have not been resolved. CTHRC1 might therefore control remodeling as either an osteoclast product or a signal within the osteoblast lineage.

As indicated above, several studies have shown that osteoclast-containing cultures secrete products (such as S1P and CTHRC1) that promote osteoblast differentiation. This approach is not without its pitfalls. One difficulty is the technical challenge of obtaining sufficiently purified osteoclasts. With present methods it is not possible to prepare osteoclasts free of macrophage content. Furthermore, the extensive overlap in gene expression between osteoclasts and macrophages suggests that factors produced by osteoclasts are also produced by macrophages, which can also be found in the BMU. Activities identified in conditioned media as osteoclast derived are therefore very often produced not only by osteoclasts but also by macrophages (to which they are closely related) and, indeed, they are often produced by other local bone cells (see **Table 1**). Could macrophages or partially differentiated osteoclasts also produce coupling factors? This is a possibility: In v-ATPase V_0 subunit D2-deficient mice, increased bone formation was observed even though osteoclast precursors could not fuse to form osteoclasts; this suggested that coupling factors may be produced by the osteoclast precursors, which were abundant (57). These considerations do not call into question the potential importance of these factors but illustrate that the coupling process is complex, involving multiple cell types, and may include cross-regulation of the pathways involved.

Table 1 provides a list of candidate factors, including those discussed above, that have been suggested as osteoclast-derived coupling factors. It should be noted that few of these have been shown to influence remodeling through in vivo studies, and it is not yet known whether these osteoclast products influence the osteoblast lineage at different stages of differentiation (**Figure 2**), although it is likely that most factors synthesized by the osteoclast would influence only the earliest stages of osteoblast precursor recruitment in the BMU (**Figure 3*b***).

FACTORS EXPRESSED ON THE OSTEOCLAST CELL MEMBRANE

More recent work has refined the concept that activity of mature osteoblasts is promoted by direct cell–cell contact with membrane-bound proteins on the osteoclast cell surface. Although this is possible when cells are studied together in culture, and may occur during bone development, such direct contact between osteoclasts and mature osteoblasts would be rare in the traditional model

of the bone modeling BMU. This is because of the reversal phase, which occurs during a time delay of several weeks between the bone resorption and formation (**Figure 1**). These mechanisms are more likely to come into play in coupling when osteoclasts come into contact with osteoblast precursors, with bone lining cells either on the bone surface or in the remodeling canopy, or even with osteoblast lineage cells in the reversal phase of remodeling (see below). Such mechanisms may also be important in other non-BMU-related contexts when osteoclasts and mature osteoblasts are in direct contact, such as in the developing calvarial suture (58).

The first example of direct cell–cell communication between osteoclasts and the osteoblast lineage proposed was ephrinB2, a ligand for the EphB4 membrane-bound receptor tyrosine kinase. EphrinB2 is expressed at all stages of osteoblast differentiation, including osteocytes, and is expressed in osteoclasts and their precursors (59–61). When osteoclasts and osteoblasts were cultured together, such in vitro contact initiated bidirectional signaling between the cells: Osteoclast-derived ephrinB2 contacts EphB4 in the osteoblast, thereby introducing forward signals that stimulate bone formation, while promoting osteoclast differentiation in the osteoclast by reverse signaling through the ephrinB2 ligand (61). However, mice with osteoclast lineage-specific ephrinB2 deletion demonstrated no bone phenotype (59, 61), nor did osteoclast precursors from such a mouse show altered osteoclast differentiation (59). Instead, pharmacological inhibition of EphB4/ephrinB2 signaling and genetic deletion of ephrinB2 in the osteoblast lineage showed that the ephrinB2/EphB4 system has an antiapoptotic role within the osteoblast lineage required for normal osteoblast differentiation (59) and, in osteocytes, it limits bone mineralization (62). Since bone formation requires extensive contact among osteoblasts (63, 64), in the BMU, such membrane-bound proteins are more likely to act in this process within the osteoblast lineage rather than communicating between two cell types that are rarely in contact.

Another contact-mediated mechanism between osteoblasts and osteoclasts proposed to regulate bone formation is the interaction between semaphorin D (Sema4D) and its receptor PlexinB1. Sema4D is stimulated in cultured osteoclast precursors by RANKL, and PlexinB1 is expressed on the cell surface of osteoblasts (65). This interaction is particularly interesting because it proposes a mechanism by which osteoclasts could inhibit osteoblast differentiation in the BMU. Female Sema4D-null mice had more osteoblasts and bone formation and normal osteoclast numbers, leading to high bone mass (65). Consistent with this, Sema4D inhibition using either antibody- (65) or siRNA-based (66) approaches increased bone mass and stimulated bone formation in ovariectomized mice. The high bone mass of Sema4D-null mice was essentially reproduced both by transfer of Sema4D-null marrow to wild type mice and by global PlexinB1 receptor deletion (65). This led to the conclusion that Sema4D is an osteoclast-derived inhibitor of osteoblast differentiation and bone formation (i.e., a negative coupling factor). This suggests it is a fine-tuning inhibitor of remodeling in the BMU. The increase in bone formation observed may also result from changes in blood supply to the bone surface, because Sema4D also induces angiogenesis via Plexin B1 (67). As mentioned above for other osteoclastic proteins, there are a range of sources of Sema4D in the BMU in addition to osteoclasts such as T lymphocytes, where it is expressed in both soluble and membrane-bound forms (68). Rather than direct contact between osteoclasts and osteoblasts (or their precursors) within the BMU, these other routes may also mediate Sema4D's inhibitory action on bone formation.

A further membrane-bound coupling activity that has recently come to light is outside-in or reverse signaling within osteoblasts by RANKL. RANKL is an essential factor for osteoclast formation, which is produced by the osteoblast lineage, and it signals through RANK in osteoclast precursors to promote osteoclast formation (69). This mechanism was uncovered when it was found that a RANKL-binding agent that blocked osteoclast formation also increased bone formation in vivo and promoted osteoblast differentiation in vitro (70). The latter effect was

prevented by knockdown of RANKL in the target osteoblastic cells, suggesting it was RANKL signaling within osteoblast precursors that was responsible (70). This was confirmed in an animal model of inflammatory arthritis where the RANKL-binding agent both blocked bone resorption and stimulated bone formation (71). Understanding such mechanisms is particularly important now that anti-RANKL therapy is becoming more commonly used for osteoporosis therapy. More recent data have indicated that, although reverse signaling by RANKL is a membrane-bound activity, it may not be mediated through cell–cell contact but by release of RANK-expressing extracellular vesicles (EVs) from the osteoclast; this is discussed in the next section.

THE POSSIBILITY OF VESICULAR COUPLING FACTORS

Cells express a range of membrane-containing EVs, including (from smallest to largest) exosomes, microvesicles, and apoptotic bodies (72). EVs are released by exocytosis from the cell and can both interact with surface receptors of target cells and transport intracellular components, including proteins, lipids, messenger RNAs (mRNAs), and microRNAs, to the cytosol of the target cell through endocytosis. The target cell may be adjacent, or the EVs could be transported to more distant locations, including possible release into the circulation. EV transport of membrane-bound RANK and microRNAs may represent additional coupling mechanisms within the BMU.

A study investigating effects of inflammation at the implant–bone interface indicated that EVs isolated from lipopolysaccharide-stimulated monocyte cultures could be internalized by stromal cells and thereby mildly stimulated mRNA levels of *Runx2*, an osteoblast commitment gene; this raised the possibility that locally derived exosomes might provide a mechanism to stimulate bone formation (73). Shortly after, osteoclasts were reported to release EVs (74): Electron microscopy revealed exosomes shed in cell cultures containing both osteoclast precursors and differentiated osteoclasts. A small proportion of these EVs was enriched for RANK on their surface and inhibited osteoclast generation in vitro. More recently, such vesicles containing RANK, secreted from maturing osteoclasts, have been found to increase bone formation by promoting RANKL reverse signaling to activate *Runx2* (75). This would suggest that reverse RANKL signaling in the early osteoblast lineage could be promoted by EVs enriched for RANK on their cellular membrane. Interesting possibilities are raised by this. If RANK-containing vesicles contribute a coupling mechanism, this would require controlled delivery from osteoclasts early in the life of a BMU to appropriate targets in the osteoblast lineage (**Figure 3**), as proposed for TGF-β (19) and IGF-I (20) (see section above titled Factors Released by Osteoclast-Mediated Resorption of the Bone Matrix).

The earlier study on monocytes suggested their EVs may contain miRNAs (73), small noncoding RNAs of ~22 nucleotides that, when internalized, can regulate gene expression in the recipient cell by binding corresponding mRNAs. miRNAs have been implicated as significant regulators of osteoblast and osteoclast differentiation for some time, although whether they acted within the osteoblast or osteoclast or acted via microvesicles shuttling them to other cells was not known at the time (for a review, see 76). In a microarray study, 13 miRNAs were identified in osteoclast-derived EVs, with the majority being upregulated in EVs released from RANKL-induced osteoclasts (77). Among them, miR-214-3p had been previously shown to inhibit osteoblast differentiation in vitro and bone formation in vivo through studies of mice with targeted overexpression of this miRNA and osteoclasts derived from them (78). Notably, miRNA-containing microvesicles were released by osteoclasts cultured on plastic, suggesting that bone resorptive activity is not necessary for their release. Curiously, it was suggested that the key mechanism by which such osteoclast-derived exosomes might act was not local action but one where the exosomes were released into the serum (78). Targeting to osteoblasts is proposed to be achieved by high levels of ephrinA2 expression on the EVs that bind to the EphA2 receptor in

osteoblasts (77), though this receptor is also expressed in osteoclast precursors (79), calling into question the specificity of such targeting. Further, as mentioned above for secreted proteins, miR-214 also promotes osteoclast formation (80), so it has multiple potential roles within the BMU.

Although it is appealing that osteoclast-derived microvesicles could regulate osteoblast function, either positively in the case of RANK content (75) or negatively in that of miRNA content (78), it remains important to establish how this could be controlled. What stimulates the release of these vesicles from the osteoclast? There are a few possibilities. Osteoclasts certainly release membrane-enclosed vesicles from their ruffled border into the resorptive pit; this may include their release as exosomes into the local environment. It is also possible that osteoclasts release apoptotic bodies when they undergo apoptosis at the end of the resorptive phase. Do these membrane-bound vesicles also have the ability to drive coupling? How do the vesicles gain access to their target cells, presumably osteoblast precursors, and how are the delivered amounts regulated?

THE EFFECT OF THE RESORPTION PIT

The way in which osteoclasts send coupling signals is not limited to matrix release, secretion of signaling molecules, or release of microvesicles. Osteoclasts also signal by leaving behind a resorptive pit after the completion of resorption. Osteoblast lineage cells, once they have been attracted to the resorbed bone surface, can sense changes in topography. When rat calvarial cells were cultured on bone slices with crevices, either made by osteoclasts or mechanically excavated grooves, the cells made bone preferentially in those defects, filling them exactly to a flat surface (81). This suggests that while molecules may be required to attract cells to the surface, it is the topography of the bone itself that tells them what to do. In this way, osteoclasts control osteoblast activity from a distance by establishing the size and shape of the resorptive pit to be filled. Once the formation process is established, the participating cells themselves must also sense the spatial limits, and inform each other of when the space has been filled through chemical communication. This may involve gap junctions or cell contact–dependent communication processes between bone-forming osteoblasts (82).

Because these in vitro studies used bone lacking osteocytes (81), the osteocytes are not necessary for osteoblasts to respond to topographic clues, at least in vitro. However, they may play a different role during refilling of pits left by osteoclasts in vivo. Through their fluid-filled lacunocanalicular network of communicating channels osteocytes sense and respond to mechanical strain. This highly complex communication system (83) might provide an additional coupling mechanism. Osteocytes would sense the increased strain resulting from weakening of the bone as resorption progresses (84) and respond by producing a signal to halt resorption. They would also detect when the strain is relieved as the resorbed pit is refilled by osteoblasts. Such a strain-based model for coupling was proposed in the early 1990s (85). As our understanding of osteocyte signaling increases, possible mediators are coming to light, given that osteocytes produce many paracrine factors. They could transmit signals to inhibit bone formation, such as sclerostin (3), or to stimulate formation, such as oncostatin M (46) or PTHrP (86); there are many possible candidates. Clearly, many steps are required to achieve precision in the coupling process: precursor attraction, differentiation, and extent and shape of bone formed.

A CELLULAR CANOPY AS A MECHANISM TO PROMOTE COUPLING

One question about the range of coupling factors produced or released by osteoclasts is whether bone remodeling occurs in an environment separated from other cells within the bone marrow space. This appears to be the case. A cellular canopy that forms during initiation of remodeling

and extends over the active BMU was proposed first by Rasmussen & Bordier (87). It was almost 30 years before the bone remodeling compartment (BRC) was identified in human biopsies by Hauge et al. (9). In that paper, it was suggested that lining cells lifted from the bone surface at the start of the remodeling cycle and formed a separate compartment that moved with the osteoclast during the remodeling cycle; the identity of the canopy cells as osteoblast lineage cells was confirmed by immunohistochemical markers more recently (88).

The BRC was proposed to also include sinusoidal endothelial cells and form part of the circulatory system (6). This connection of the vasculature with the BRC provided a route for osteoclast precursors, including partially differentiated quiescent osteoclast precursors (89). Capillaries associated with the canopy also provide a mechanism for ingress of other cells, including mesenchymal precursors (90) and immune and endothelial cells (**Figure 3*a***). The canopy has also been demonstrated in rabbit bone (91) and has been observed above bone-forming surfaces in the mouse (92). Tissue-specific macrophages (osteomacs) have also been found to form a canopy over bone-forming sites in the mouse (93), but it remains unclear whether these are also found at remodeling sites and what the nature of the relationship between the two canopies may be.

It has been suggested that the canopy not only forms the separate BRC but is also required for completion of the reversal phase. This stems from the observation in biopsies from osteoporotic patients with incomplete canopies at sites of reversal phase arrest; these are sites of uncoupling where bone formation is not observed following bone resorption (94, 95). Perhaps the canopy serves to keep local coupling factor concentrations sufficiently high to allow precursor recruitment or to stimulate osteoblast differentiation and bone formation. In this BRC, osteoblast lineage cells, osteoclasts, endothelial cells, vascular cells, and immune cells might exchange factors and influence precursors provided by the associated vasculature (88); very recently it has been suggested that neuronal cells may also come into close contact with the canopy at active remodeling sites (96). It has also been proposed that the osteoblast lineage cells comprising the canopy might contain target cells for coupling activity (97); for example, this might be a mechanism by which membrane-bound osteoclast-derived factors may make contact with osteoblast precursors or even the canopy cells themselves. In such a case, the contribution to the coupling process would be the signal to bone lining cells, in contact with the osteoclast, to lift the canopy (**Figure 3*a***).

Defining the canopy's contribution to the coupling process using genetically altered mouse models is limited because this anatomical structure is rarely observed in the mouse, the model that has been used most extensively for defining intercellular signaling pathways involved in bone remodeling. One major unanswered question is the mechanism by which the lifting of the canopy occurs. What signal causes it to occur? Some possibilities are that it could be driven by the formation and attachment of the osteoclast to the bone surface, or the lining cells may receive a signal to lift and make space, or actively signal, for the osteoclast to attach to the bone surface (78–80, 82).

OSTEOCLASTS AND THEIR INFLUENCE ON COUPLING DURING THE REVERSAL PHASE

The reversal phase is the period between bone resorption and bone formation (11) (**Figure 1**) and has also been postulated to mediate coupling, but the details as to how this might be achieved have been elusive. Toward the end of resorption, mononuclear cells were identified at the bottom of resorption pits, where they remove demineralized collagen to prepare the pits for the engagement of osteoblasts to form bone (98). The mononuclear cells ascribed this function were thought to be macrophages for many years. However, when their ultrastructure was analyzed, it was discovered that they are bone lining cells, and they activate matrix metalloproteinases to clean collagen

remnants from the resorption pits. Bone formation occurred only in the pits that had already been cleaned (99).

The finding that these were osteoblast lineage cells raised the question about whether they might become activated to become matrix-producing osteoblasts, as has been observed on bone surfaces in mice treated with PTH (100). This was confirmed when in situ hybridization and immunohistochemistry were used in BMUs in human trabecular and Haversian (cortical) bone (101, 102). This showed a continual progression in cellular morphology and marker expression from lining cells near the osteoclasts to plump, active osteoblasts near bone-forming surfaces (101). This thus suggests a reversal phase during which osteoblast differentiation continues until a critical mass of mature osteoblasts is reached to be followed by matrix formation (101) (**Figure 3*d***).

A further novel observation was the identification of smaller than usual osteoclasts sparsely distributed among the osteoblast lineage cells on the reversal phase surface. Their number decreased with the distance from the cortical resorption pit, likely indicating a reduction in numbers in time after resorption (**Figure 3*c***). It appears likely that these osteoclasts can signal to the nearby osteoblast lineage cells, using any of the mechanisms described above: matrix-derived protein release, protein secretion, EV release, and importantly, given their likely contact with osteoblast lineage cells, membrane-bound protein expression. The scattered distribution of osteoclasts on the reversal surface might make any membrane-associated signaling a minor component, but they could secrete activities that contribute to the osteoblast differentiation that appears to be taking place there (102).

The discovery of osteoclasts within the reversal phase complicates the simple model of bone remodeling. It was thought that apoptosis of resorbing osteoclasts would provide the signal for reversal cells to enter the BMU, but it seems it is not so simple, and there is no sudden evacuation of osteoclasts from the bone surface. This very recent finding has not yet been confirmed by others, nor observed in other species, but it is very intriguing and provides a reasonable model to explain how the initiation of bone formation may be influenced by the reversal phase.

CONCLUDING REMARKS

We now have a very different image of the BMU (**Figure 3**). While initially it was viewed as a site with a changing cast of homogenous cell types that enter, act, and leave, we now view it as a site in which heterogenous cell types mingle and carry out activities that require their interactions, as follows: (*a*) for initiation of remodeling, the lining cells lift and osteoclasts attach; (*b*) during resorption, osteoclasts resorb and signal to osteoblast precursors in preparation for the next phase; (*c*) in the reversal phase, osteoclasts and osteoblast lineage cells work together to tidy up after resorption and prepare the cells that will carry out bone formation; (*d*) finally, the osteoblasts, working together with the embedding osteocytes, form new bone matrix that is gradually mineralized. Through each of these stages, other cells within the BMU, including T cells and macrophages, also likely contribute to remodeling.

Clearly, there are many factors coordinating these processes and many mechanisms by which such coordination must take place: Now is the time to move beyond the phase of merely identifying what must be many potential coupling factors. We must spend time defining the types of mechanisms by which these factors drive the process of remodeling, and identifying the aspects of those mechanisms that can be useful for intervening in human skeletal diseases.

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