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Novel Insights into the Coupling of Osteoclasts and Resorption to Bone Formation

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Abstract

Bone remodeling consists of resorption by osteoclasts (OCs) and formation by osteoblasts (OBs). Precise coordination of these activities is required for the resorbed bone to be replaced with an equal amount of new bone in order to maintain skeletal mass throughout the lifespan. This coordination of remodeling processes is referred to as the “coupling” of resorption to bone formation. In this review, we discuss the essential role for OCs in coupling resorption to bone formation, mechanisms for this coupling, and how coupling becomes less efficient or disrupted in conditions of bone loss. Lastly, we provide perspectives on targeting coupling to treat human bone disease.

Keywords

Osteoclast; coupling factor; resorption; bone remodeling; bone formation

1. Introduction

The adult skeleton undergoes bone remodeling to maintain mineral homeostasis and bone quality [1,2]. Bone remodeling consists of resorption by multi-nucleated, myeloid-derived osteoclasts (OCs) followed by formation by mesenchymal-derived osteoblasts (OBs)[3]. OCs and OBs are organized together with canopy, bone lining, and reversal cells within the basic multicellular unit (BMU)[4–6]. The term BMU helps to describe the sequential steps of bone remodeling that occur over several weeks as one individual, coordinated process [4,7]. Precise coordination of these activities is necessary to ensure replacement of bone at sites of resorption and the overall maintenance of skeletal mass. This coordination of

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bone remodeling activities is commonly referred to as the coupling of bone resorption to bone formation[8]. With aging and other conditions, such as gonadal hormone loss, the rate of resorption exceeds formation resulting in reduced bone mass [9]. In other pathological conditions, resorption occurs independently from subsequent bone formation leading to destructive bone loss[7]. A better understanding of the mechanisms coupling resorption to bone formation may lead to novel therapeutic strategies to maintain skeletal mass throughout the lifespan and potentially drive anabolic bone accrual in conditions of severe bone loss.

In this review, we focus specifically on the mechanisms by which OCs and resorption are coupled to subsequent bone formation. In addition, we highlight emerging areas of interest, including how the coupling of resorption to formation is impacted by systemic energy metabolism and inflammation. Lastly, we provide perspective on the therapeutic targeting of coupling mechanisms to treat human bone diseases.

2. OCs couple bone resorption to bone formation

The essential role for OCs in coupling resorption and bone formation was largely elucidated through the study of osteopetrosis. Osteopetrosis, which translates to “stone bone,” is a rare disease characterized by increased bone mass resulting from a lack of OC resorption[10,11]. Osteopetrosis patients exhibit an accumulation of bone density, which leads to altered bone structure and bone fragility. While there are several different forms of osteopetrosis, with variable degrees of clinical manifestations and severity, it can be broadly classified into one of two categories: OC-poor and OC-rich. OC-poor osteopetrosis patients exhibits few OCs due to mutations in genes required for normal OC differentiation, such as *TNFSF11* and *TNFRSF11A*, which encode for Receptor Activator of NF-kappa-B Ligand (RANKL) and its receptor RANK, respectively[11]. These patients also have reduced OB numbers and bone formation[12]. In contrast, OC-rich osteopetrosis patients exhibit normal or increased numbers of OC that have impaired resorption capacity due to mutations in genes required for the generation of the characteristic OC ruffled border and acidification of the resorption lacunae (e.g. *TCIRG1, CLCN7, OSTM1*, and *PLEKHM1)[11].* In contrast to OC-poor, OC-rich osteopetrosis patients exhibit normal or increased OBs and bone formation markers[13,14], showing that the presence of OCs even without functional resorption contributes to OB differentiation and bone formation.

Similarly to in humans, mutations in mice that inhibit OC differentiation or resorptive function leads to OC-poor and OC-rich osteopetrosis phenotypes, respectively. The *op/op* mouse model of osteopetrosis results from a spontaneous loss of function mutation to *Csf1*, the gene encoding macrophage-colony stimulating factor (M-CSF)[15]. M-CSF is required for macrophage and OC differentiation, and *op/op* mice lack OCs and bone resorption. Similar to OC-poor osteopetrosis patients, *op/op* mice exhibit reduced OBs and mineralization [16]. On the other hand, the *oc/oc* osteopetrosis mouse model results from spontaneous mutation to *Tcirg1*, which encodes a subunit of the vacuolar proton pump required for acidification of the resorption lacunae. Similar to *TCIRG1* mutation in humans, the *oc/oc* mice exhibit normal or increased numbers of OCs; however these OCs have impaired bone resorption activity[17]. While *oc/oc* mice die 3–4 weeks after birth, transplant of fetal-liver derived hematopoietic stem cells from *oc/oc* mice into wild type

adult mice leads to a significant reduction in bone resorption indices, but increased bone formation rate [18]. In addition to these spontaneous models of osteopetrosis, OC-poor and -rich phenotypes can be modeled through targeted deletion of specific genes required for OC differentiation or resorption, respectively [19,20].

The coupling-related effects of modulating OC numbers and/or function is also evident following treatment with pharmacologic anti-resorptive therapies. The biotherapeutic Denosumab (DMAb) is a neutralizing antibody to human RANKL that blocks OC differentiation and function[21], and drives fission of multinucleated OCs[22], overall leading to decreased OCs. Bisphosphonates (BPs) are small molecule anti-resorptive drugs that reduce the number of functional OCs on the bone surface through a variety of mechanisms [23], including effects on OC differentiation and maturation [24] and apoptosis. In addition, certain BPs also impair OC G-protein signaling and cytoskeletal functions, leading to the presence of detached OCs within the bone marrow compartment [25]. While these anti-resorptive therapies effectively reduce bone loss, they are linked to a coupling-related reduction in OBs and bone formation [26–29]- although there are also possible direct effects of certain BPs to inhibit new bone formation [29]. The coupling-related reduction in OBs following anti-resorptive therapy is also associated with blunted induction of bone formation by bone anabolic parathyroid hormone (PTH) [30–33].

On the other hand, odanacatib is an anti-resorptive drug that inhibits the OC-specific protease cathepsin K (CTSK), thus inhibiting resorption-mediated protein degradation without impairing other OC processes [21]. Loss of function mutations to *CTSK* in humans results in pycnodystosis, which has many similarities to OC-rich osteopetrosis[34]. Inhibition of CTSK activity via odanacatib does not reduce OCs, and unlike BPs and DMAb, OB numbers and bone formation are sustained [35,36]. Despite positive effects on bone mass, odanacatib was pulled from development during phase 3 trials due to an unexpected increase in cardiovascular events[37]. Regardless, odanacatib provides proof-of-concept pharmacological data that inhibition of resorption while maintaining functional OCs protects bone formation. Further understanding of the mechanisms linking OCs and resorption to subsequent bone formation may lead to the development of new anti-resorptives that protect and/or stimulate bone formation.

3. Mechanisms coupling bone resorption to bone formation

Several mechanisms have been proposed for the coupling of OCs and resorption to OB-mediated bone formation, and it is highly likely that multiple mechanisms act together to coordinate optimal coupling (Figure 1A). These mechanisms involve local, paracrine signals derived from OCs or bone matrix acting on cells within the bone remodeling compartment (BRC). The majority of identified coupling mediators are soluble factors; however, membrane coupling factors have also been identified. In addition to proposed coupling factors, optimal coupling may also depend on extracellular matrix (ECM) signals and surface topologies exposed or created during bone resorption.

3.1 Soluble factors

Soluble factors, either released from the bone matrix during resorption or secreted by OCs (Figure 1B), were the first mechanisms proposed for the coupling of resorption and formation, and these coupling factors have been reviewed extensively[7,38–40]. In one of the earliest studies investigating coupling, Howard, et al., demonstrated that PTH-induced acute resorption was followed by an extended period of bone formation. Following dialysis of conditioned media (CM), the authors revealed the presence of soluble factors larger than 12,000 Daltons derived from bone and/or OCs. CM from the resorbing cultures also stimulated bone formation in separate organ cultures[41]. While this work did not result in the identification of specific coupling factors, it provided evidence for OC resorption derived soluble factors that mediate the coupling of resorption to bone formation.

Transforming Growth Factor (TGF)-β was one of the first specific soluble factors identified coupling resorption to bone formation. Bone matrix is rich in TGF-β protein[42], in particular TGF-β1[43], which is bound with Latency Associated Peptide (LAP) and Latent TGF-β Binding Protein (LTBP)[44]. OCs release and activate TGF-β during bone resorption[45]. Matrix-derived TGF-β was first shown to act as a coupling factor by Tang, et al., who documented a low bone formation phenotype in *Tgfb1* null mice that was driven by the loss of TGF-β-induced OB progenitor migration[46]. However, TGF-β also has known inhibitory effects on bone formation, suggesting additional coupling factors must promote OB differentiation and bone formation[46,47]. A second matrix derived protein released during resorption is Insulin Growth Factor 1 (IGF1)[47]. Deletion of *Igf1r* in OB lineage cells reduced bone formation, consistent with a role for matrix IGF1 in coupling resorption to formation. Xian, et al., further demonstrated that IGF1 signaling in OB progenitors activated mammalian target of rapamycin (mTOR) signaling and OB differentiation to drive bone formation at the site of resorption[47].

Subsequently it was proposed that matrix-derived TGF-β may also act directly on the OC lineage to induce the expression of coupling factors[48]. Treatment of mature OCs with TGF-β1 induces gene expression of several secreted factors, including *Cxcl16*[49], *Lif*[49,50], *Sphk1*[48], *Wnt1*[51], and *Wnt10b*[48] that have been linked to bone formation and/or coupling[52–63]. Of these genes, we confirmed induction of *Wnt1* in OCs cultured on murine bone chips. Induction of *Wnt1* gene expression was blocked by treatment with a TGF-β receptor inhibitor, confirming that the effects are mediated through TGF-β signaling and not an indirect effect of bone matrix[51]. TGF-β signaling activates SMAD2/3 phosphorylation; phosphorylated SMAD2 or SMAD3 bind to SMAD4, leading to nuclear translocation and regulation of gene expression. In contrast, bone morphogenetic proteins (BMPs), which belong to the TGF-β superfamily, activate SMAD1/5/8 phosphorylation. Phosphorylated SMADs bind to SMAD4 to translocate to the nucleus and regulate gene expression[64]. In the presence of TGF-β and BMP, SMAD2/3 and SMAD1/5/8 compete for available SMAD4. In this capacity, SMAD1/5 activation has been shown to antagonize OC coupling factor expression[65]. Thus, it is possible that resorption-derived TGF-β drives coupling factor expression to promote bone formation; as OBs differentiate, the concentration of BMPs increases and antagonizes TGF-β-induced coupling factor expression to limit bone formation.

While matrix derived TGF-β and IGF1 provide mechanistic links between resorption and bone formation, it is well established that OC-rich osteopetrosis patients with impaired resorption exhibit normal or increased bone formation[11,13,14]. This has been recapitulated in several mouse models of OC-rich osteopetrosis[20,66], as well as transplant models in which hematopoietic cells from OC-rich mice[67], but not OC-poor, increases bone formation[68]. Further, CM generated from OCs on plastic promotes OB differentiation and bone nodule formation *in vitro*, although less potently than OCs on bone [69,70]. Thus, OCs must secrete signals to promote OB differentiation independently of resorption.

Several gp130 signaling members have been identified as potential OC-derived coupling factors. The first of these was cardiotrophin (CT)-1. CT-1 is expressed by OCs, and deletion of CT-1 results in large OCs with reduced resorptive activity; in contrast to OC-rich osteopetrosis models, CT-1 deficient mice exhibit reduced bone formation. Treatment with CT-1 promotes OB differentiation and bone formation, consistent with a potential role as an OC-derived coupling factor[71]. In further support for gp130 members in coupling OCs to OBs, Fernandes, et al., found that cord blood-derived OCs promote bone formation, and this effect was ablated by neutralization of gp130 or OncostatinM (OSM)[72]. Similarly, Leukemia Inhibitory Factor (LIF), an Interleukin-6 family cytokine also secreted by OCs, drives increased bone formation[52,53]. Adult LIF knockout mice have decreased bone formation with no significant changes to OC numbers[73]. Our genetic analysis of human bone needle biopsies from placebo and DMAb treated participants revealed that *LIF* expression correlates with OC and OB coupling at the gene expression level, and *LIF* was significantly reduced in bone biopsies isolated from DMAb treated participants, consistent with a potential role in coupling[74]. In addition to directly stimulating OB progenitor migration and mitogenic activity[49,53], LIF, CT-1, and OSM may activate bone formation indirectly through modulation of osteocyte (OT) gene expression. Walker, et al., showed that treatment of the UMR 106–01 OT-like cell line with LIF, CT-1, and OSM downregulate expression of sclerostin, a Wnt signaling inhibitor[61]. Consistent with this, Koide, et al., revealed that OC-CM downregulates OT sclerostin, and this effect is mediated via LIF[75]. Mutations to the sclerostin gene, *SOST*, are associated with the high bone mass diseases sclerosteosis and van Buchem disease[76]; downregulation of this soluble inhibitor of Wnt signaling may thus increase Wnt-induced bone formation. Thus, OC-derived factors may also couple bone resorption to bone formation indirectly through OTs.

Another OC-derived factor proposed to couple resorption and bone formation is sphingosine-1-phosphate (S1P). S1P is a sphingolipid that is involved in several cell functions, including migration, differentiation, and apoptosis[77]. S1P is produced by sphingosine kinase (Sphk)-1 and −2. OCs have been shown to express SPHK enzymes and produce S1P to promote OB migration and survival[56,62]. A role for S1P in coupling was supported by Lotinun, et al., who showed that deletion of *Ctsk* in the OC lineage increases bone formation through elevated OC lineage S1P production[57]. However, the role for S1P in bone remodeling is multifaceted, including an essential role in OC precursor migration from blood vessels to bone[78,79]. Treatment of mice with S1P receptor agonist[79] or an inhibitor of S1P lyase, which increases S1P by inhibiting its degradation[80], both improve bone mass; however, these positive effects appear to be through an anti-resorptive

mechanism, since mice exhibit significant reductions in OCs[79,80]. It is important to note that the mice maintain bone formation despite the reduction in OCs. Thus, while the primary function for S1P in remodeling may be as a regulator of OCs, increasing S1P may support OB and prevent a coupling-related decrease in bone formation.

In addition to OC-derived factors acting on the OB lineage, OCs also promote bone formation by driving angiogenesis to the bone remodeling compartment. Blood vessels have long been recognized as a component of the BMU[4]. These vessels deliver essential metabolic nutrients for remodeling and provide a source of stem cell progenitors for OB differentiation. Several reports demonstrated a specific role for type H vessels ($CD31^{hi}Emcn^{hi}$) in supporting osteogenesis[81,82]. Subsequently, Xie, et al., demonstrated that OC-lineage secreted Platelet-Derived Growth Factor (PDGF)-BB induces endothelial progenitor cell migration to drive angiogenesis of $CD31^{hi}Emcn^{hi}$ vessels to support bone formation[83]. Conditional knockout of *Pdgfb* in the OC-lineage resulted in lower bone mass, along with reduced $CD31^{hi}Emcn^{hi}$ vessels compared to wildtype. $CD31^{hi}Emcn^{hi}$ vessels were also reduced in an ovariectomy (OVX) mouse model of post-menopausal bone loss; $CD31^{hi}Emcn^{hi}$ vessels and bone formation were rescued by exogenous PDGF-BB or CTSK inhibition, which increased pre-OC derived PDGF-BB. In addition to effects on angiogenesis, Xie, et al.[83], and others have demonstrated PDGF-BB also drives chemotaxis of mesenchymal OB progenitors[84,85]. OC lineage expression of *PDGFB* has now been reported in human bone sections, along with expression of PDGF receptors *PDGFRA* and *PDGFRB* by mesenchymal lineage canopy and reversal cells adjacent to resorbing OCs[86]. OC-lineage expression of PDGFB appears to be downstream of G protein-coupled receptor kinase 2 interacting protein (GIT)-1, as knockout of GIT1 leads to reduced bone formation and $CD31^{hi}Emcn^{hi}$ vessels through reduced pre-OC PDGF-BB[87]. *PDGFB* expression is also induced by TGF-β[88], suggesting a role for matrix-derived TGF-β in activating this coupling activity.

Other proposed OC-derived soluble coupling factors include BMPs[89], collagen triple helix repeat containing 1 (CTHRC1)[90], Complement 3a (C3)[91], and SLIT3[92]. Of interest, many of these are induced by treatment with TGF-β, as discussed above. This suggests that matrix-derived TGF-β may polarize OC lineage cells towards a “coupling” phenotype, leading OC to express several bone anabolic proteins concentrated within the BRC; this phenotype may then be antagonized by increasing OB-derived BMPs to prevent excess bone formation[65]. However, several of these soluble factors may be present at a level sufficient for driving coupling activity, even in the absence of bone resorption activity. In that case, increasing the presence of non-resorbing OC-lineage cells promotes coupling activity.

3.2 Membrane factors

In addition to soluble factors, cell-cell interactions between OCs and mesenchymal lineage cells via membrane factors have been proposed as mechanisms for coupling resorption to bone formation. Cell-cell interactions between OC and OB lineage cells are an established mechanism for the OB lineage to stimulate of OC differentiation. OB lineage cells express membrane bound RANKL, which activates RANK on the OC lineage cell membrane. However, resorption and formation are spatially and temporally restricted during the bone

remodeling cycle[6], suggesting that OC membrane factors more likely contribute coupling-effects on reversal and/or canopy cells[7].

The first cell-cell interaction proposed for coupling of resorption to formation was the EphrinB2-EphB4 membrane interaction[93]. Zhao, et al., demonstrated OC and OB expression of *Efnb2* and OB expression of *Ephb4*. The authors used antibodies to activate bidirectional signaling in OCs and OBs. However, myeloid lineage specific knockdown of EphrinB2 did not impact the bone phenotype, suggesting that EphrinB2 from other cells, or other Ephrin family members, compensates in the absence of OC EphrinB2[93]. Further study revealed that OB EphrinB2 activates anti-apoptotic signaling in neighboring OBs to drive bone formation[94]. Given evidence that OCs do express EphrinB2 *in vivo*[95], it remains possible that this cell-cell interaction contributes to anti-apoptotic effects in the OB lineage during the reversal phase.

The second membrane factor proposed for coupling resorption to formation via cell-cell interactions is semaphorin4D (Sema4D). OC lineage Sema4D activates PlexinB1 on OBs. Rather than promoting coupling, Sema4D acts as a negative regulator of OB differentiation, and knockout of either Sema4D or PlexinB1 increases bone formation and improves skeletal mass. Neutralizing antibody to Sema4D delivered either prophylactically or therapeutically similarly improved bone mass in OVX mice by increasing bone formation[96]. In humans, serum Sema4D positively correlates with markers of resorption and negatively correlates with bone mineral density in postmenopausal osteoporosis[97].

The most recent observation of membrane interactions coupling OCs and OBs is reverse signaling through the established signaling partners RANK and RANKL. Reverse signaling via RANKL is the signaling pathway activating within RANKL expressing cells following interaction with OC lineage RANK. Furuya, et al., utilized a peptide (W9) to block RANKL-induced OC differentiation and found that W9 induced bone anabolic effects *in vivo*. *In vitro* studies confirmed that this was dependent on OB RANKL expression, suggesting reverse signaling via RANKL in OBs[98]. Reverse RANKL signaling could also be activated via treatment with soluble RANK[99] and this effect was dependent on a proline-rich motif in the RANKL cytoplasmic tail. Mutation of a proline residue within this motif to alanine (Pro29Ala) prevented remodeling-induced bone formation in two models of elevated bone turnover without affecting resorption[100].

Importantly, activation of RANKL reverse signaling does not require cell-cell interactions, as RANK expressed on OC-derived extracellular vesicles (EVs) can also activate reverse signaling in RANKL expressing OBs[100–102]. EVs are membrane-derived vesicular bodies that are released into extracellular space[103]. EVs provide a mechanism for cells to communicate with both neighboring and distant cells, allowing communication of spatially restricted OCs and OBs during bone remodeling. Ikebuchi, et al., documented RANK protein on EVs derived from RAW 264.7 OC-like cells. These RANK positive EVs stimulated OB gene expression in OB cell lines and primary cultures. *In vivo* administration of an inhibitor of EV secretion or an antibody against a murine OC-derived EV marker (immunoglobulin superfamily member 8, IGSF8) both prevented remodeling induced bone formation, demonstrating that OC derived EVs may promote bone formation[100].

There are several subtypes of EVs classified by origin, function, and/or biogenesis. Three main classes of EVs are exosomes, microvesicles, and apoptotic bodies. Exosomes and microvesicles exhibit lipid bilayers and range in diameter from 40–120 and 50–1,000nm, respectively[103]. Exosomes more closely reflect the membrane composition of their cell of origin and exhibit a biogenesis mechanism distinct from microvesicles. Ikebuchi, et al., showed that RANK positive EVs co-precipitated with exosome markers CD9, CD63, and CD81, suggesting that these could in fact be OC-derived exosomes[100]. Apoptotic bodies (ABs) are a larger subtype of EV, ranging in diameter from 500–2,000nm, generated via blebbing of an apoptotic membrane[103]. Ma, et al., used flow cytometry to separate EVs based on size, with exosomes and microvesicles distinct from ABs[104]. Through separation of these populations, Ma, et al., demonstrated that OC-derived ABs, but not BMM or pre-OC ABs, had increased RANK content and increased ability to drive OB differentiation *in vitro* compared to BMM, pre-OC, and OC-derived exosomes and microvesicles. In both studies[100,104], the effects on OB differentiation were blocked through the addition of soluble RANKL which competes with OBs for binding the RANK positive EVs.

In addition to activating reverse signaling through RANKL, Liang, et al., revealed that OC-derived small EVs (~90nm size) were enriched with four miRNAs: miR128–3p, miR-324, miR-130b-ep, and miR-1187. Through *in vitro* overexpression in OB cell line, they found that miR-324 activated, while miR-130b-3p inhibited OB differentiation. Thus, in addition to activation of reverse RANKL signaling, RANK-RANKL membrane interaction can target microRNAs and other EV content specifically to the RANKL positive OB lineage cells to regulate OB differentiation and bone formation[105].

In contrast to the report by Liang, et al.[105], two additional studies showed that OC-derived EVs contain miR-214 and inhibits of osteogenesis[106,107]. Mice transgenic for OC-derived miR-214 exhibited reduced bone formation and bone mass; further, osteoporosis patients exhibited increased serum exosome miR-214[106,107]. Treatment with a miR-214 antagomir promoted bone formation in aging mice[106]. While these results are report a negative effect of OC-derived EVs on OB differentiation, they similarly identified OC-OB membrane interactions that may mediate the targeting of OC-derived EVs to OBs. Li, et al., show that OC-derived EVs are positive for Sema4D, and OB uptake of these EVs could be blocked via anti-Sema4D antibody[106]. Sun, et al., demonstrated an Eprhin-mediated mechanism for targeting OC-derived EVs to OBs[107]. Thus, while the overall effect of OC-derived EVs on bone formation remains uncertain and may be context dependent, the membrane factors mediating OC-OB interactions likely play a crucial role in specifying signaling cargo for target cells. The potential for OC-derived EV also extend the role for membrane factors in coupling beyond the spatial and temporal restrictions of these cellular interactions within the BRC.

3.3 Resorption effects on bone matrix

Bone is composed of both organic and inorganic extracellular matrix (ECM) that act as a physical scaffold and provide mechanical and biochemical signals to regulate cell proliferation, adhesion, and differentiation[108–110]. Changes to bone ECM through either deletion of organic protein components *in vivo* or *in vitro* modifications to scaffold content

and/or surface topography has a major impact on bone formation[108]. OC resorption alters the bone ECM and surface topography[111–113]. Because of the role for ECM and surface topography in regulating OB differentiation, it is likely that OC resorption-induced changes to the bone matrix contributes to the propensity for OB to differentiate and form bone at resorption sites.

The role of surface topography in regulating OB differentiation and bone formation has been thoroughly evaluated in the context of osteointegration which depends on optimal interactions between biomedical devices and host tissue. Several studies have shown that surface roughness regulates OB proliferation, adhesion, and differentiation, with OB-like cells exhibiting decreased proliferation and increased differentiation on rough surfaces[114–117]. Animal and clinical studies support these findings, showing that rough surfaces improve long-term integration of implants as compared to smooth surfaces[118–120]. Of interest, a combination of micron and sub-micron surface roughness results in three-dimensional topographies similar to the surfaces created by OC resorption during normal remodeling[111,112,121]. Culturing of OBs on dentine or bone chips shows that osteoprogenitors preferentially differentiate and form bone at sites of mechanical defects or prior resorption[112,113], suggesting that surface properties generated may contribute to coupling of resorption to formation.

While performing gene expression analyses of human bone biopsies to identify potential OC-derived coupling factors[74], we stumbled upon a link between the bone matrix and coupling. We performed RNA-sequencing of centrifuged human bone biopsies from participants treated with a single dose of placebo or DMAb, and identified OC and OB signatures that were significantly reduced in DMAb-treated biopsies. Rank means of the OC and OB signatures showed a high degree of correlation, demonstrating OC-OB coupling at the gene expression level[74]. Although not part of our study, we postulated that correlation of OC and OB signatures across the entire expression dataset may reveal novel pathways associated with resorption and formation, respectively. As a proof-of-concept, we ran a correlation analysis of the OC and OB gene sets across a comprehensive list of 555 bone genes, compiled from bone-related gene ontology (GO) gene lists. Genes that significantly correlated with OC or OB signatures were then evaluated by iPathway Analysis, using the positive or negative slope of the correlation in place of fold change. We reasoned that pathways overlapping between OC and OB signatures are pathways involved in coupling. Further, pathways also downregulated in DMAb biopsies, in which patients exhibit a coupling-related decrease in bone formation[21,26,27], may represent specific pathways mediating the coupling of resorption and formation (Figure 2).

In all three groups, ECM-receptor interaction was the most significantly correlated pathway. While more experiments are needed, these data support that altered ECM-receptor signaling may be crucial for coupling. It is possible that with DMAb treatment, the significant reduction in OCs and resorption leads to reduced exposure of ECM and surface topographies required for optimal OB differentiation and bone formation. However, it is important to note that patients with OC-rich osteopetrosis that also lack resorption pits still show bone formation[11]. Thus, the bone surface created within the resorption pit is not a sole

requirement but part of a multifaceted regulatory system together with OC lineage-derived soluble and membrane factors to promote bone formation at sites of bone resorption.

4. Importance of energy metabolism for maintaining coupling

Bone formation, along with bone remodeling as a whole, is energy expensive. Altered energy availability and impaired nutrient sensing are linked to reduced bone mass and quality and increased fracture risk. Direct inhibition of these pathways in OBs lead to reduced OB number and bone formation[47,122,123]. Thus, OB cell energetics is a central to the ability of these cells to respond to OC and resorption-derived coupling signals.

Evaluation of bone cell energetics has provided insights into the importance of energy availability and nutrient sensing for normal bone formation responses. Esen, et al., showed that Wnt3a-induced OB differentiation was accompanied by a metabolic shift to aerobic glycolysis[124], or the “Warburg effect,” defined by preferential conversion of glucose to lactate even in the presence of oxygen[125]. Aerobic glycolysis has previously been shown to occur in bone explant studies[126,127], with anabolic PTH inducing lactate production, further supporting a role for aerobic glycolysis in bone formation[128–130]. More recent studies have indicated a role for oxidative phosphorylation in OB differentiation; however, differentiated OBs are more glycolytic than undifferentiated OBs[131]. Of interest, Wnt-induced bone formation was disrupted by knockdown of glycolytic enzymes[124]. Since Wnt signaling has been shown to contribute to coupling, this raises the question of how coupling is impacted by energy availability and nutrient sensing.

We and others have recently proposed RANKL-RANK signaling as a mechanism linking bone remodeling to energy metabolism. Bonnet, et al., show that RANKL drives peripheral insulin resistance in muscle, resulting in increased serum glucose; in contrast to muscle, RANKL increases bone glucose uptake. The effects of RANKL to drive peripheral insulin resistance and increased bone glucose uptake were prevented by DMAb neutralization of RANKL[132]. Consistent with this, pre-diabetic or diabetic patients treated with DMAb, but not calcium/vitamin D or BPs, showed improved glucose control[74]. Given that RANKL drives bone remodeling, this suggests that the peripheral insulin resistance increases glucose availability for the high energy demanded for bone remodeling. OCs were also positive for dipeptidyl-peptidase (DPP)-4, which degrade incretins leading to reduced insulin secretion and sensitivity[74]; this suggests an additional mechanism by which OCs promote peripheral insulin resistance to increase glucose availability.

Glucose is a major energy source for osteoblasts[133–135]. In addition to serving as an energy source, glycolysis provides metabolic intermediates for other metabolic pathways and the biosynthesis of proteins, lipids, and nucleotides. It is also possible that glycolysis provides intermediates for generation of bone matrix. Of interest, several of these energy and biosynthetic pathways are controlled via the Phosphoinositide 3-kinase (PI3K)-Akt signaling pathway.

In evaluating pathways correlating with OC and OB bone biopsy signatures and downregulated by DMAb, we also identified PI3K-Akt signaling pathway as a potential

mediator of coupling (Figure 2). There is substantial evidence for PI3K-Akt signaling pathway in skeletal development and bone remodeling. Elevated PI3K-Akt signaling at specific stages of OB lineage leads to aberrant or increased OB differentiation[136,137] and/or increased bone formation[138]. The PI3K-Akt pathway is activated by and/or interacts with bone anabolic pathways including insulin, IGF-1 and Wnt and BMP signaling pathways[139]. Several of these factors have been identified as OC or resorption-derived coupling factors[47,48,51,56]. While OCs also exhibit a high energy demand during resorption, the impact of impaired OB nutrient sensing on bone formation along with the convergence of OC and resorption-derived coupling factors on the PI3K-Akt signaling pathway highlight the importance of OB cell energetics in the coupling of resorption to formation.

5. Impact of inflammation on coupling

Despite extensive mechanisms coordinating the coupling of resorption to bone formation, the rate of resorption exceeds bone formation with aging and certain disease conditions. Moreover, conditions such as rheumatoid arthritis (RA) and bone infections exhibit resorption independent of bone formation. Given the increase in inflammation with aging, and the prominent role for inflammation in RA and bone infections, inflammation likely contributes to imbalanced resorption and formation.

Aging is associated with increased formation of aggressive, trench-forming OCs[140]. Separate studies revealed increased inflammatory OCs in autoimmune disease models[141,142], and a role for inflammasome signaling in driving resorption[143,144]. Trench forming OCs exhibit increased CTSK activity[145,146] and may itself be a population of inflammatory OCs. Given the increased acidification and CTSK activity exhibited by trench forming OCs[146], it is likely that the resulting resorption pits exhibit altered surface topographies, and potentially less optimal surfaces for OB differentiation. In addition, inflammatory OCs may exhibit altered secretomes. Thus, it seems likely that inflammatory and/or trench-forming OCs have an altered propensity to couple bone resorption to bone formation; however, this remains to be shown.

The BMU vasculature is a source of immune cells that can directly influence bone remodeling and the coupling of resorption and formation[38]. T cell involvement in bone remodeling has been well-established, and T cells can directly regulate OCs. T cells can express RANKL to induce OC differentiation and resorptive activity[147]. T cells isolated from RA patients were positive for RANKL, and other inflammatory factors, and stimulated OC differentiation[148]. In addition, interferon (IFN)-y, another T cell-derived cytokine, has inhibitory effects on OC. Disruption of T cell IFN-y signaling is associated with osteoporosis, osteomyelitis, and RA[149]. Not only can T cells induce OC activation and potentially activate aggressive resorption, T cells can also serve as a source of Wnt inhibitors to inhibit OB differentiation. Recently, T cell derived Dickkopf (DKK)-1 (Wnt inhibitor of Dickkopf-1) was shown to contribute to OVX-induced bone loss[150].

In addition to inflammatory immune populations, aging and certain diseases are associated with senescent cell accumulation that contribute to age-related pathologies[151]. Cellular

senescence is defined as a terminal growth arrest in response to cell stress, such as DNA damage or oncogene activation. Senescent cells produce a senescence-associated secretory phenotype (SASP) composed of inflammatory factors such as IL-6 and TNF[151]. We identified senescent cells within the aging mouse skeleton[152], and showed that the SASP promotes OC progenitor survival and increased OC differentiation. Inhibition of the SASP or neutralization of SASP components prevented OC induction, and OCs were similarly reduced in a genetic model of senescent cell ablation. Of interest, senescent cell ablation reduced OCs, but increased bone formation[153]. This suggests that either SASP-induced OCs do not effectively couple resorption to bone formation (e.g. inflammatory subset of OCs) or alleviation of the SASP improves OB progenitor function. Related to altered OB progenitors, a recent report highlighted that aged skeletal stem cells have decreased bone forming potential, and increased propensity to drive inflammation and resorption[154]. Thus inflammation driven by aging and/or senescent cells alters OCs and OBs functions consistent with impacts on the altered coupling of resorption and formation.

5. Conclusions

OCs and bone resorption are coupled to subsequent bone formation during bone remodeling. Given the positive effects of OCs on bone formation even in the absence of resorptive activity, there is much interest in developing novel anti-resorptives to block bone degradation without reducing OCs. Odanacatib was pulled from development due to off-target risk for cardiovascular events[37]. However, if the CTSK inhibition could be localized to bone-resorbing OCs, it remains an effective strategy for reducing resorption without affecting OC number. In addition, targeting CTSK in animal models increases OC progenitors leading to increased OC-lineage derived coupling factors that protect bone formation[57,83]. As an alternative, pharmacologic agents that act on the OC lysosome, such as hydroxychloroquine[155], may also be effective to reduce CTSK protein maturation and acidification of the resorption lacunae.

The discovery of RANK positive EVs[100,104] offers the possibility of developing exosome-based therapies to promote bone formation. RANK positive exosomes or EVs could aid in the delivery of exogenous miRNAs, pharmacologic agents, or proteins specifically to RANKL positive OB progenitors. In such a case, it is theoretical that coupling activated bone formation may be possible even in the absence of OCs.

Further elucidation of the mechanisms by which OC couple resorption to bone formation, and how this is impacted by systemic energy metabolism and inflammation, may lead to novel therapeutic strategies to maintain bone mass or to stimulate bone accrual in conditions of severe bone loss. Conversely, these coupling mechanisms may also serve as targets to prevent increased bone mass in osteopetrosis.

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Abbreviations

AB	apoptotic body
BMP	bone morphogenetic protein
BMU	basic multicellular unit
BP	bisphosphonate
BRC	bone remodeling compartment
CM	conditioned media
CT	cardiotrophin
CTSK	Cathepsin K
DMAb	denosumab
EV	extracellular vesicle
IGF	insulin growth factor
LIF	leukemia inhibitory factor
OB	osteoblast
OC	osteoclast
OSM	oncostatin M
OT	osteocyte
PDGF	platelet derived growth factor
PTH	parathyroid hormone
RANK	Receptor Activator of NFkB
RANKL	RANK ligand
Sema	semaphorin
TGF	Transforming growth factor

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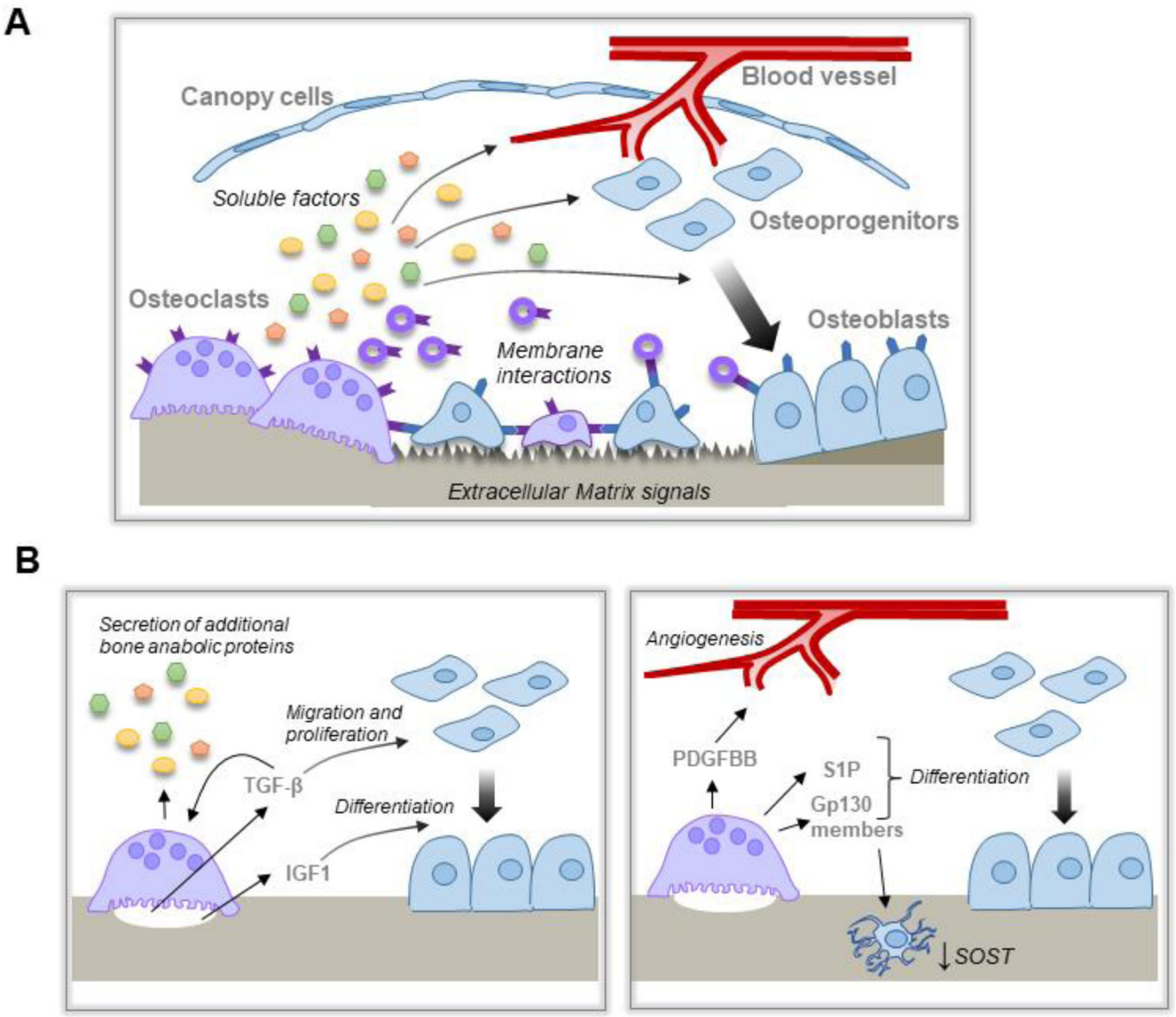

Figure 1. Mechanisms by which OCs couple bone resorption to bone formation.

A, Schematic overview of the proposed mechanisms by which OC and resorption are coupled to bone formation. OC resorption releases matrix derived and OC-derived soluble factors and/or EVs that act on multiple cell lineages within the BRC to promote bone formation. OC membrane factors, including on EVs, interact with cognate receptors on cells within the BRC. In addition, altered bone matrix promotes optimal bone formation at sites of bone resorption. B, Matrix derived factors (left panel) released during bone resorption stimulate differential effects on osteoblast migration and proliferation vs differentiation. Matrix derived TGF-β also induces expression of other bone anabolic proteins. OC expression of additional factors (right panel) induce angiogenesis, osteoblast differentiation, and indirect effects on bone formation through the osteocytes.

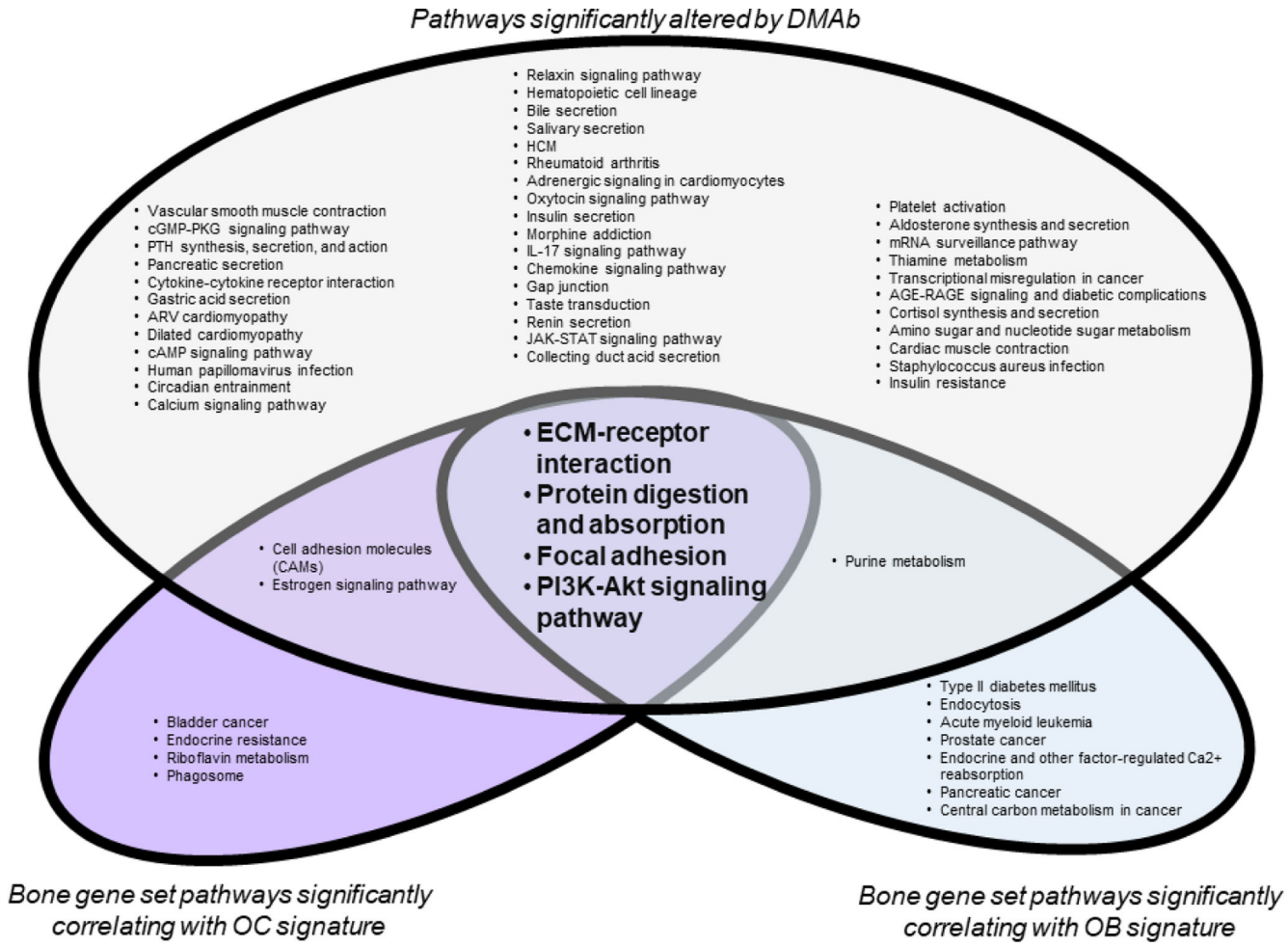

Figure 2. Pathways mediating the coupling of bone resorption and bone formation.

Centrifuged human bone biopsies were subjected to RNA-sequencing (GSE141614). 555 bone genes were compiled from gene ontology bone gene sets. From this, OC and OB genes downregulated by DMAb were identified. Rank means of the OC and OB gene signatures, respectively, were used to assess correlation across the comprehensive bone gene set. Significant differential gene expression (DMAb vs placebo) or correlation with OC or OB gene signatures is defined as $p<0.05$. Significant genes were analyze by iPathway Analysis Software (Advaita) to identify pathways significantly affected by DMAb treatment (blue), or pathways correlating with OC (red) or OB (green) gene signatures. Significant pathways are defined as $p<0.10$. Pathways significantly associated with DMAb differential gene expression and OC and OB gene signatures may indicate pathways associated with the coupling of OC and OB.