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Osteoclast-derived coupling factors and exosomal packaging microRNA regulate bone formation and remodelling

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ABSTRACT

Bone remodelling is a continuous process by which bone resorption by osteoclasts is followed by bone formation by osteoblasts to maintain skeletal homeostasis. These two forces must be tightly coordinated not only quantitatively, but also in time and space, and its malfunction leads to diseases such as osteoporosis. Recent research focusing on the cross-talk and coupling mechanisms associated with the sequential recruitment of osteoblasts to areas where osteoclasts have removed bone matrix have identified a number of osteogenic factors produced by the osteoclasts themselves. Osteoclast-derived factors and exosomal containing miRNA can either enhance or inhibit osteoblast differentiation through paracrine and juxtacrine mechanisms, and therefore may have a central coupling role in bone formation. Entwined with angiocrine factors released by vessel-specific endothelial cells and perivascular cells or pericytes, these factors play a critical role in angiogenesis-osteogenesis coupling essential in bone remodelling.

***Key words*:** bone remodelling, osteoblasts, osteoclasts, bone microenvironment, exosomal microRNA, coupling factor, angiogenesis, osteogenesis

CONTENTS

I. INTRODUCTION

The skeleton is a metabolically active organ that undertakes constant remodelling throughout life to maintain its structural integrity and calcium homeostasis. Bone remodelling relies on the accurate balance between bone resorption by osteoclasts and bone matrix formation by mesenchymal lineage osteoblasts, and involves a complex series of sequential steps that are highly regulated. The group of cells actively participating in remodelling is designated as the basic multicellular unit (BMU) (Kular, Tickner, Chim *et al.*, 2012; Sims & Walsh, 2012). The cellular activities in each BMU aims to achieve a correct balance between osteoblast and osteoclast activity in response to osteoclastic bone resorption, designated "coupling", to maintain a balanced bone mass. When this coupling is interrupted, the accurate bone mass could be compromised, leading to skeletal disorders such as osteoporosis and osteopetrosis. A greater understanding of the mechanisms underlying coupling between the osteoclast and osteoblast coordination in bone remodelling may open a new avenue to identifying target molecules for alternative therapies more efficacious against these bone disorders.

The BMU consists of cells that contribute signalling pathways to bone resorption (osteoclast) or bone formation (osteoblast), and include osteocytes, T-cells, macrophages, pericytes, vascular endothelial cells, canopy bone lining cells, and precursor populations of osteoblasts and osteoclasts (Kular *et al.*, 2012; Sims *et al.*, 2012) (Fig. 1). Bone remodelling is known to be regulated by both local and systemic factors, which include parathyroid hormone (PTH), calcitriol, hormones (such as thyroid hormone, growth hormone, sex hormone, and glucocorticoids), and growth factors (such as insulin-like growth factors (IGFs), tumour growth factor-beta (TGF-beta), bone morphogenetic proteins (BMP), and prostaglandins). Furthermore, it is well established that osteoblastic like cells govern the

formation and function of osteoclasts through the osteoprotegerin (OPG) / receptor activator of NF-kappa B ligand (RANKL) / RANK axis (Kong, Yoshida, Sarosi *et al.*, 1999).

Emerging evidence points to the involvement of factors derived from osteoclasts themselves in the coupling process (Sims & Martin, 2014a; Sims & Martin, 2015). For instance, studies have found that complete inhibition of both osteoclast formation and function by Denosumab, a humanised anti-RANKL antibody, leads to the reduction of bone formation secondary to reduced osteoblast activity because of the loss of osteoclast-derived coupling factors that serve to stimulate bone formation (Kostenuik, Nguyen, McCabe *et al.*, 2009) . Further, the inhibition of both osteoclast formation and function by long-term bisphosphonate use is linked to the occurrence of osteonecrosis of the jaw (ONJ) (Reyes, Hitz, Prieto-Alhambra *et al.*, 2016) or atypical sub-trochanteric femoral fractures (Miller & McCarthy, 2015) due to its adverse effects on bone remodelling, resulting from decreased bone formation. In comparison, inhibition of bone resorption function without affecting its differentiation by Odanacatib (ODN), a selective and reversible inhibitor of cathepsin K, does not cause the reduction of bone formation. In fact, bone formation was preserved at certain skeletal sites (Sims & Ng, 2014b). It appears that ODN prevents the degradation of matrix-derived proteins in supernatants of osteoclasts, including IGF-I, and BMP-2 (Fuller, Lawrence, Ross *et al.*, 2008). This preservation of bone formation appears to be due to the effects of coupling factors secreted by osteoclasts and released from demineralised bone matrix. This indicates that bone resorptive activities of osteoclasts are separable from their coupling activities.

This review aims to provide an exploration of coupling factors and mechanism of cross talks between the osteoclast and osteoblast, and to relate their functionality to uncoupling consequences in pathological bone and joint diseases. The regulatory mechanisms of osteoblast-directed osteoclastic bone resorption is well documented, however there is

accumulating evidence to indicate that osteoclasts are also conversely controlling osteoblastic bone formation. Dissecting the molecular mechanisms that regulate the function of coupling factors in bone remodelling will give insight into potential future therapies for these bone diseases.

II. THE BONE REMODELLING UNIT AND CYCLE

Bone remodelling takes place within anatomically distinct sites within the skeleton termed basic multicellular unit (BMU) which comprises a tightly regulated cohort of cells (Kular *et al.*, 2012; Sims *et al.*, 2012) (Fig. 1). During this process unwanted or damaged bone is resorbed by osteoclasts and replaced with new bone by osteoblasts at the approximately same location, to maintain bone mass at the same level during adult life. This is distinct from bone modelling where bone formation occurs at sites that have not been marked by osteoclastic resorption, resulting in a transformation in the size, shape or micro-architecture of the bone.

Bone remodelling is a multicellular event involving osteoprogenitors on the bone surface, capillary blood supply, mesenchymal envelope like canopy cells surrounding the bone marrow, and within the bone matrix itself (Fig. 1). These multiple cell types communicate or cross talk with each other, and are essential for proper bone development and homeostasis (Schipani, Wu, Rankin *et al.*, 2013). Evidence indicates that the growth of blood vessels and activity of vascular endothelial growth factor (VEGF) in bone and osteogenesis are also coupled, and is regulated by different capillary subtypes (Clarkin & Gerstenfeld, 2013; Kusumbe, Ramasamy & Adams, 2014). Also recruitment of osteoprogenitors from the canopy onto reversal surfaces is important during bone remodelling (Jensen, Andersen, Hauge *et al.*, 2015).

The bone remodelling cycle is accomplished according to three distinct sequential phases (Fig. 2).

(1) Initiation Phase

During the *Initiation Phase*, there is recruitment of osteoclast precursors to the BMU from hematopoietic precursors, and differentiation of osteoclast precursors into multinucleated osteoclasts. Osteoblasts control of osteoclast differentiation and activation is regulated by osteoblast - expressed molecules such as RANKL, OPG (Sims *et al.*, 2014a; Sims *et al.*, 2015), and Semaphorin 3A (Hayashi, Nakashima, Taniguchi *et al.*, 2012).

(2) Reversal Phase

A *Reversal Phase* then emerges whereby osteoclast function is subdued via apoptosis or autophagy whereas osteoblast linage cells are recruited and differentiated. The reversal phase is a transition from osteoclast bone resorption to osteoblast bone formation. There appears to be a gap or delayed period between bone resorption and formation (Sims *et al.*, 2014a; Sims *et al.*, 2015), and coupling mechanisms are employed to overcome this time delay (Fig. 2). Osteoclast-derived factors can directly or indirectly (being refined by other factors) initiate the differentiation of osteoblasts in resorbed sites to form new bone. Other anabolic coupling factors are also released from the resorbed bone matrix and other cell types within the BMU, such as TGF-beta and bidirectional signalling between EphrinB2 on osteoclasts and EphrinB4 on osteoblast precursors might facilitate this transition phase.

(3) Termination Phase

The *Termination Phase* involves osteoblastic bone formation and mineralization of the bone matrix (Raggatt & Partridge, 2010). The estimated average length of the remodelling phase in human cancellous bone from iliac crest is about 3 weeks for bone resorption and 3-4 months for bone formation (Eriksen, Gundersen, Melsen *et al.*, 1984; Eriksen, Melsen & Mosekilde, 1984), and approximately 5 weeks for reversal phase (Tran Van, Vignery & Baron, 1982). However, the period of the bone remodelling process varies by species and type of bone, and is influenced by the disruption or perturbation of coupling activities in

pathological conditions such as such as osteoporosis, bone tumors, rheumatoid arthritis, and osteoarthritis.

III. OSTEOCLAST DERIVED COUPLING FACTORS

The concept of "coupling" in the bone remodelling process was first proposed by Frost's group who observed sites of osteoclastic bone resorption was sequentially replaced by osteoblastic bone formation (Hattner, Epker & Frost, 1965). The increase in bone formation (due to increased number of osteoclasts) observed with the impairment of bone resorption due to a deficiency of the chloride channel ClC-7 or c-src kinase activity (Marzia, Sims, Voit *et al.*, 2000; Schaller, Henriksen, Sveigaard *et al.*, 2004), further suggest that osteoclasts exhibit coupling mechanism to promote osteoblast activity independently of their bone resorbing activity. It is proposed that during osteoclastic bone resorption, coupling factors are produced by osteoclasts to regulate osteoblast activity in the BMU (Table 1). To date, four major classes of osteoclast-derived coupling factors have been reported, which include matrix-derived factors released during osteoclastic bone resorption, osteoclasts secreted factors, osteoclast membrane-bound molecules, and osteoclast-derived exosomal microRNAs (miRNAs) (Fig. 3).

(1) Matrix- derived factors

A number of latent form of growth factors are imbedded into bone matrix during matrix formation, and reactivated during the next cycle of osteoclastic bone resorption (Oreffo, Mundy, Seyedin *et al.*, 1989) (Fig. 3A). These proteins which include TGF-beta (Bonewald & Mundy, 1990; Crane & Cao, 2014) , BMP-2 , platelet-derived growth factor (PDGF) (Tsukamoto, Matsui, Fukase *et al.*, 1991; Xie, Cui, Wang *et al.*, 2014a), and IGFs (Mohan & Baylink, 1996) are also activated by plasmin generated by plasminogen activators (Campbell,

Novak, Yanosick *et al.*, 1992; Yee, Yan, Dominguez *et al.*, 1993) and matrix-metalloproteinases (Dallas, Rosser, Mundy *et al.*, 2002). Howard et al. was one of the earliest to propose that resorption was accompanied by the release of growth factors stored in the bone matrix which contribute to the coupling activities and restoration of bone loss in the BMU (Howard, Bottemiller, Turner *et al.*, 1981). More recently Ota et al. demonstrated that TGF-beta1 induces Wnt10b production in osteoclast to enhance coupling to osteoblasts (Ota, Quint, Ruan *et al.*, 2013a), and CXCL16 and LIF which modulate recruitment of osteoblasts during bone remodelling (Ota, Quint, Weivoda *et al.*, 2013b). In addition, mouse genetic experiments have shown that TGF-beta1 promotes migration of mesenchymal stem cells (MSCs) in bone microenvironment that facilitate the coupling of bone resorption with formation (Tang, Wu, Lei *et al.*, 2009). IGF-1 was also found to promote recruitment of MSCs and osteoblast differentiation (Xian, Wu, Pang *et al.*, 2012). In bone microenvironment, dysregulation of these locally produced factors can contribute to disease condition and progress. For example, dysregulation of TGF-beta alters the fate of MSCs, leading to the bone microarchitecture damage characterised in rheumatoid arthritis (Crane *et al.*, 2014). In bone metastasis, TGF-beta can contribute to a vicious cycle of bone metastasis and bone loss (Juarez & Guise, 2011).

(2) Osteoclast – secreted factors: a paracrine mechanism

A sets of secrete factors are produced by osteoclasts, and via a paracrine mode of action, promote osteoblast migration, differentiation, and bone formation in the BMU (Fig. 3B). These secreted factors act as a ligand and bind to their receptors on osteoblasts: cardiotrophin-1 binds with pg130 receptors (Sims & Walsh, 2010; Walker, McGregor, Poulton *et al.*, 2008); sphingosine-1-phosphate (S1P) with S1P1 and S1PR2 receptors (Quint, Ruan, Pederson *et al.*, 2013; Ryu, Kim, Chang *et al.*, 2006); BMP-6 with their specific type-I

and type-II serine/threonine kinase receptors BMPR-I and BMPR-II (Pederson, Ruan, Westendorf *et al.*, 2008); Wnt10b and Dickkoph-related protein 1 (DKK-1) with Frizzled receptors and their co-receptors low-density lipoprotein receptor-related protein -5 (LRP5) or LRP6 (Ota *et al.*, 2013a); CTHRC1 with the Wnt-Fzd/Ror2 receptor complex (Takeshita, Fumoto, Matsuoka *et al.*, 2013; Yamamoto, Nishimura, Misaki *et al.*, 2008), and complement factor 3a (C3a) with the C3a receptor (Matsuoka, Park, Ito *et al.*, 2014). Secreted afamin derived from nonresorbing osteoclast - derived functions as chemokine for preosteoblasts migration via the regulation of Akt-signaling pathway (Kim, Lee, Lee *et al.*, 2012). In contrast, the serine protease HTRA1 (Wu, Chim, Kuek *et al.*, 2014) is a secreted factor released by osteoclasts, and inhibit osteoblast differentiation, suggesting that osteoclasts could produce both negative and positive factors to modulate bone remodelling process.

(3) Osteoclast membrane bound factors: a juxtacrine mechanism

Recent studies indicate that osteoclasts and their precursors can regulate osteoblast formation and functions by means of direct cell-cell contact via cell-surface regulatory proteins (Fig. 3C). EphrinB2 protein expressed by osteoclasts as a cell surface molecule can interact with its receptor EphB4 in osteoblasts and regulate osteoblast differentiation. Interestingly, the EphB4 receptor can signal to EphrinB2 in the reverse direction to suppress the formation of osteoclast precursors (Zhao, Irie, Takada *et al.*, 2006). Thus the bidirectional signalling between the cell-surface ligand EphrinB2 and its receptor EphB4 is important in the regulation of bone absorption and remodelling. Dis-regulation of ephrinB2/EphB4 axis is seen in osteolytic disorder such as multiple myeloma (Pennisi, Ling, Li *et al.*, 2009).

Semaphorin 4D (Sema-4D) is an osteoclast derived molecule that can act through its receptor Plexin-B1 on osteoblasts to inhibit bone formation (Negishi-Koga, Shinohara, Komatsu *et al.*, 2011). Antibody treatment specific to Sema-4D markedly prevented bone

loss in a model of postmenopausal osteoporosis (Negishi-Koga *et al.*, 2011), and its inhibition partly counteracts alveolar bone loss caused by osteoporosis (Zhang, Wei, Miron *et al.*, 2014) and lytic skeletal metastases associated with breast cancers and other epithelial malignancies which overexpress Sema-4D (Yang, Buhamrah, Schneider *et al.*, 2016). Taken together, targeting Sema-4D might represent a new therapy to cancers - induced osteolytic conditions.

(4) Osteoclast-derived exosomal packaging microRNA

Osteoclast-derived exosomal microRNAs (miRNAs) represents a new class of osteoclast-released coupling factor. MicroRNAs are small noncoding RNA molecules containing ~22 nucleotides that regulate gene expression and versatile biological processes (Rigoutsos & Furnari, 2010). A series of miRNAs has been characterised to regulate osteoblastic bone formation, and the dysregulation of these miRNAs affects skeletal health (Lian, Stein, van Wijnen *et al.*, 2012). Exosomal-containing miR-214-3p (Li, Liu, Guo *et al.*, 2016; Sun, Zhao, Li *et al.*, 2016) are osteoclast-derived inhibitors of osteoblast differentiation and bone formation. Increased osteoclastic miR-214-3p is associated with reduced bone formation in elderly women with fractures and in ovariectomized mice (Li *et al.*, 2016) (Fig. 3D) *In vitro* and *in vivo* studies have identified that osteoclast-derived exosomal miR-214-3p can negatively impact osteoblastic bone formation, suggesting a model of paracrine action (Li *et al.*, 2016; Sun *et al.*, 2016). Thus, inhibition of osteoclast - derived miR-214-3p can promote bone anabolic action, and has therapeutic potential.

IV. OTHER LOCAL FACTORS THAT AFFECT BONE REMODELLING

Osteogenesis during bone remodelling is coupled with angiogenesis for nutrient supply and help further couple bone resorption and bone formation (Figs 1 and 2). PDGF-BB secreted by preosteoclasts stimulates proliferation and migration of both endothelia progenitor cells

(EPCs) and MSCs, and induces CD31 and endomucin (CD31(hi)Emcn(hi)) vessel formation during bone remodelling, thus coupling angiogenesis and osteogenesis (Xie, Cui, Wang *et al.*, 2014b). PDGF-BB secreted by osteoclasts influences the temporal-spatial vessel formation for new bone formation involving stabilising newly formed vessels, mobilising mesenchymal stem cells, and promoting osteoblast differentiation (Ramasamy, Kusumbe, Wang *et al.*, 2014; Xie *et al.*, 2014a). Other secreted factors of the adipogenic signaling molecules such as leptin, and adiponectin could also affect bone microenvironment by altering bone marrow adipocytes, which might lead to impaired vascular and osteogenesis (Muruganandan & Sinal, 2014).

Angiocrine factors such as Noggin mediates endothelial-cell-specific Notch pathway and osteogenesis (Kusumbe *et al.*, 2014; Ramasamy *et al.*, 2014). Further, Notch signalling in endothelial cells can regulate hematopoietic stem cell niches via CD31-positive capillaries and PDGFR-B-positive perivascular cells (Kusumbe, Ramasamy, Itkin *et al.*, 2016). Epidermal growth factor-like domain 7 (EGFL7) which is expressed in osteoclasts and osteoblasts is an antagonist to the Notch pathway (Schmidt, Bicker, Nikolic *et al.*, 2009), and regulates endothelial cell migration and angiogenesis (Chim, Kuek, Chow *et al.*, 2015; Chim, Tickner, Chow *et al.*, 2013). In addition, EGFL6 and Nephronectin (NPNT) expressed osteoblasts regulate angiogenesis by a paracrine mechanism in bone microenvironment (Chim, Qin, Tickner *et al.*, 2011; Kuek, Yang, Chim *et al.*, 2016). Interestingly, NPNT and CD31 expression is reduced in bone of ovariectomised mice and in osteoporosis patients (Kuek *et al.*, 2016). It is likely that exosomal-containing microRNA released from osteoclasts and osteoblasts could also regulate angiogenesis in the bone microenvironment. Identification of novel angiogenic and angiocrine factors that regulate trabecular bone mass and angiogenesis in bone microenvironment will be a future subject of investigation.

V. CONCLUSIONS

1. Bone is a dynamic tissue that undergoes life-long remodelling regulated by the tight coupling of bone resorption and bone formation.
2. During bone remodelling, osteoclast bone resorption and osteoblast bone formation occur independently.
3. Osteoclast-derived factors and exosomal packaging miRNA can either be inhibitory or promote osteogenesis, and emerging evidence indicates that osteoclasts regulate osteoblast bone formation independent of resorptive activity.
4. Osteogenesis in bone remodelling is coupled with angiogenesis. Factors released by preosteoclasts and osteoclasts are able to temporally and spatially coordinate angiogenesis during bone growth and remodelling.
5. It is remarkable that with so many contributors involved in the coupling process, that a genetic error of a single participant leads to pathological processes secondary to altered bone remodelling – when it might be reasonable to expect compensatory mechanisms would normalise the balance in the BMU.
6. The pathways involved in bone remodelling is far more complex than our current understanding, and new insight into coupling mechanisms will help to translate these factors into novel therapeutic approaches for the treatment of bone diseases.
7. However, much work remains to be done to fully understand the nature and significance of the individual factors involved in coupling, the interplay between the many contributors, time- and dose-specific effects of each factor, and their regulation during bone growth and pathology.

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FIGURE LEGENDS

Figure 1. Schematic diagram showing cells in the basic multicellular units cells that contribute to the bone remodelling in bone microenvironment.

Figure 2. Schematic diagram showing three phases of bone remodelling processes, including initiation phase, reversal phase and termination phase. Note that a cross-talk between osteoclasts and osteoblasts involves a wide variety of cells existing in BMU. (1) Some factors released by osteoclasts are imbedded in bone matrix and may have a direct impact on osteoblasts or regulate osteocytes to modulate the activity of bone formation at resorbing compartments. (2) A direct communication between osteoclasts and osteoblasts during the phase of bone remodeling. (3) Mesenchymal stem cells are also plausible targets for osteoclast-derived coupling factors to modulate resorbed bone formation. (4) Endothelial cells act as important intermediates to deliver coupling signals from osteoclast precursors to osteoblastic osteogenesis.

Figure 3. Schematic diagrams showing 4 common modes of coupling factors by osteoclasts that regulate osteoblast activities, including (A) matrix-derived factors released during osteoclastic bone resorption, (B) soluble factors synthesised and secreted by osteoclasts, (C) factors expressed as membrane-bound proteins on the osteoclast cell surface, and (D) osteoclast-derived exosomal microRNAs (miRNAs) that influence osteogenesis.

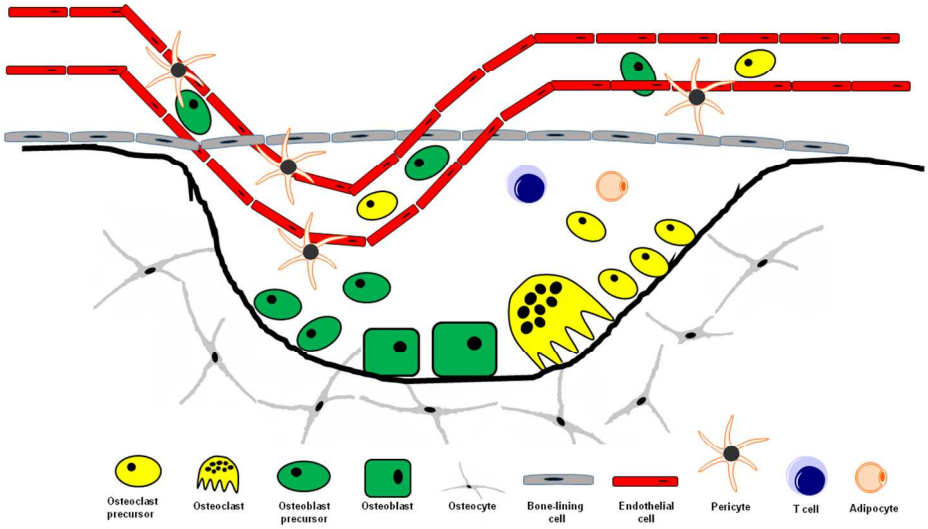

Figure 1. Schematic diagram showing cells in the basic multicellular units cells that contribute to the bone remodelling in bone microenvironment.
Figure 1
338x190mm (96 x 96 DPI)

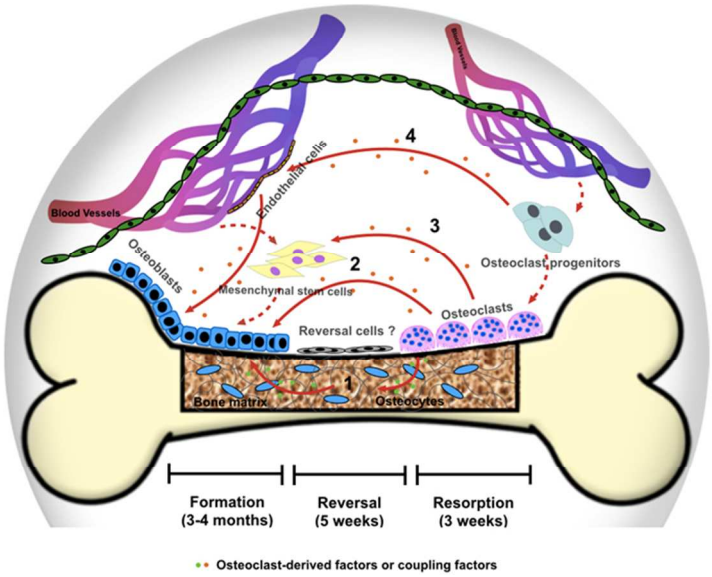

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Figure 2
254x190mm (96 x 96 DPI)

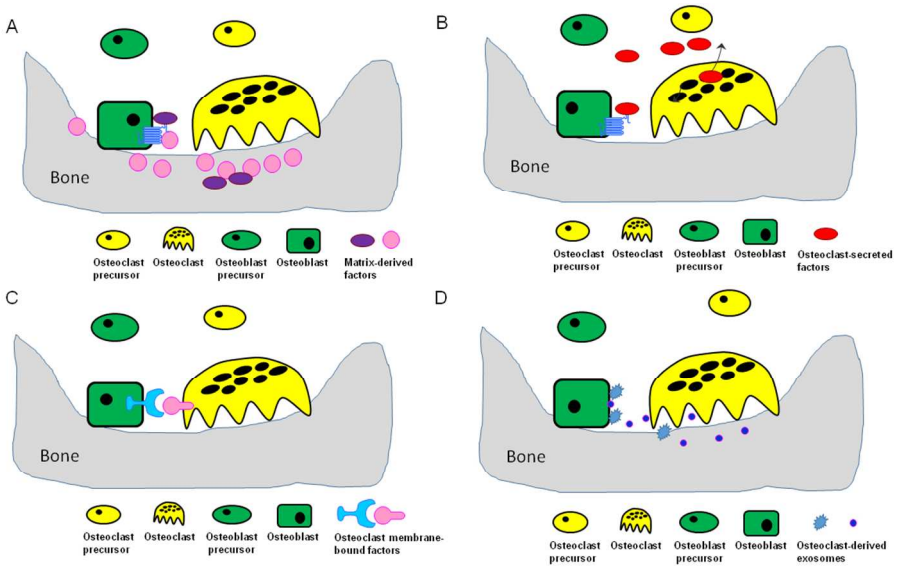

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Figure 3
338x190mm (96 x 96 DPI)

Table 1. Examples of osteoclast-derived coupling factors and their potential actions

Coupling factors	Derived from	Targeting on	Secreted or Membrane bound	Reference
TGF-beta	Bone matrix	Inhibit osteoblasts	Matrix derived secreted	(23, 24)
IGFs	Bone matrix	Promote osteoblast migration and proliferation	Matrix derived secreted	(28)
PDGF	Bone matrix	Promote osteoblast activity and migration	Matrix derived secreted	(26, 27)
Semaphorin 4D	Osteoclasts	Inhibit osteoblasts	Membrane bound	(50)
EphrinB2	Osteoclasts	Promote osteoblast differentiation	Membrane bound	(48)
Sphingosine-1-phosphate	Osteoclasts	Promote osteoblast migration and survival	Secreted	(40, 41)
BMP6	Osteoclasts	Promote osteoblast migration	Secreted	(42)
Complement component 3a	Osteoclasts	Promote osteoblast differentiation	Secreted	(45)
CTHRC1	Osteoclasts	Promote osteoblast differentiation and migration	Secreted	(43)
Afamin	Osteoclasts	Promote preosteoblast migration	Secreted	(46)
CXCL16	Osteoclasts	Promote osteoblast migration	Secreted	(34)
Wnt10b	Osteoclasts	Promote osteoblast mineralization	Secreted	(33)
Cardiotrophin-1	Osteoclasts	Promote osteoblast activity and mineralization	Secreted	(39)
HTRA1	Osteoclasts	Inhibit osteoblasts	Secreted	(47)
PDGF-BB	Preosteoclasts	Promote CD31(hi)Emcn(hi) endothelial cells	Secreted	(26)

Table 1
Table 1
182x136mm (300 x 300 DPI)