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Molecular signaling in bone cells: regulation of cell differentiation and survival

Lilian I. Plotkin[*,¥,#,1], **Angela Bruzzaniti**[§,*,¥]

[§] Department of Biomedical and Applied Sciences, Indiana University School of Dentistry, Indianapolis, IN, USA

[*] Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN, USA

[¥] Indiana Center for Musculoskeletal Health, Indianapolis, IN, USA

[#] Roudebush Veterans Administration Medical Center, Indianapolis, IN, USA

Abstract

The achievement of proper bone mass and architecture, and their maintenance throughout life requires the concerted actions of osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells. The differentiation and activity of osteoblasts and osteoclasts are regulated by molecules produced by matrix-embedded osteocytes, as well as by cross-talk between osteoblasts and osteoclasts through secreted factors. In addition, it is likely that direct contact between osteoblast and osteoclast precursors, and the contact of these cells with osteocytes and cells in the bone marrow, also modulate bone cell differentiation and function. With the advancement of molecular and genetic tools, our comprehension of the intracellular signals activated in bone cells has evolved significantly, from early suggestions that osteoblasts and osteoclasts have common precursors and that osteocytes are inert cells in the bone matrix, to the very sophisticated understanding of a network of receptors, ligands, intracellular kinases/phosphatases, transcription factors, and cell-specific genes that are known today. These advances have allowed the design and FDA-approval of new therapies to preserve and increase bone mass and strength in a wide variety of pathological conditions, improving bone health from early childhood to the elderly. We have summarized here the current knowledge on selected intracellular signal pathways activated in osteoblasts, osteocytes, and osteoclasts.

Keywords

osteoblast; osteoclast; osteocyte; bone; differentiation; apoptosis

2. INTRODUCTION

The maintenance of bone mass throughout life is attained by the regulated actions of osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells (Figure 1)

[1] Corresponding author: lplotkin@iupui.edu.

(Bellido et al., 2014). Osteoblasts are cuboidal cells present on the bone surface and are derived from mesenchymal stem cells (MSCs). Osteoblasts produce and secrete components of the bone matrix and regulate the deposit of hydroxyapatite, the mineral component of the mature bone. Osteoclasts are multinucleated cells derived from hematopoietic precursors also present on the bone surface. Osteoclasts produce enzymes that work in an acid environment and are able to dissolve bone mineral and proteins such as collagen molecules, removing bone and allowing for new bone to be formed. At the same time, osteoclastic bone resorption results in the release into the circulation of factors such as TGFβ, which are stored in bone and can now act on target cells and tissues.

Osteoblasts and osteoclast differentiation and activity are under the control of osteocytes, former osteoblasts embedded in the mineralized bone matrix (Bonewald, 2011). Over the past decade, our understanding of osteocyte biology has expanded and it is now clear that these cells are producers and targets of extracellular molecules that modulate cell activity and survival. In addition, all bone cells, as well as cells present in the bone marrow and in other tissues, produce factors that trigger intracellular signaling and modulate bone formation and resorption. Thus, a well-controlled network of cytokines and growth factors participate in the regulation of bone homeostasis. In addition to this paracrine/autocrine regulation, the generation, proliferation and survival of bone cells is regulated by systemic hormones, including parathyroid hormone, sex steroids (both androgens and estrogens) and glucocorticoids, reviewed elsewhere (Zuo and Wan, 2017, Wein and Kronenberg, 2018, Hachemi et al., 2018). In this chapter, we will describe the signaling pathways triggered in osteoblasts, osteocytes, and osteoclasts by selected regulatory molecules, as well as by direct cell-to-cell contact. The intracellular signaling mechanisms activated by additional bone-acting stimuli, as well as the biological consequences of these signaling pathways have been recently described in (Plotkin and Bivi, 2014, Verlinden et al., 2016, Sims, 2016, Strazzulla and Cronstein, 2016, Grafe et al., 2018, Lisowska et al., 2018, Thompson et al., 2012).

3. OSTEOBLASTS AND OSTEOCYTES

Osteoblasts, which originate from MSC precursors, are the cells responsible for the synthesis of bone matrix proteins to form osteoid, and for matrix mineralization, to form mineralized, mature bone (Figure 1) (Bellido et al., 2014). Under the influence of locally produced molecules, the expression of lineage-specific transcription factors is stimulated and MSC undergo differentiation (Bradley et al., 2018). These transcription factors include osterix, and Runx2 (Bellido et al., 2014) and deletion of these genes in mice results in defective osteoblast differentiation and lack of mineralized bone (Otto et al., 1997, Nakashima et al., 2002).

As osteoblast become surrounded by the bone matrix, they undergo changes in their morphology and the pattern of gene expression, transitioning into osteocytes, which are the most abundant and long-lived cells in bone. In spite the difference in morphology and gene expression pattern, most of the extracellular signaling molecules affect both osteoblasts and osteocytes, albeit with different biological consequences. We will describe the signaling pathways activated in both cell types, indicating when these pathways lead to different biological outcomes in osteoblast and osteocytes (Figure 2).

3.1. Bone morphometric proteins

Bone morphogenetic protein (BMPs) are growth factors that belong to the transforming growth factor (TGF) β superfamily of proteins originally identified as inducers of ectopic bone formation (Katagiri and Watabe, 2016) and are now known to exert a potent osteogenic effect by promoting osteoblast differentiation and function. BMPs are synthesized as precursors, which are later cleaved and become active. Structurally, most of the BMPs exist as covalently-bound homodimers, although heterodimers have also been found.

BMPs bind to the BMP receptor (BMPR) type I (ALK-1–7) alone or in combination with a type II receptor (BMPR-II, ACTR-IIA, or ACTR-IIB). Type I and II receptors differ in cellular expression and ability to bind particular ligands, but all share kinase activity. Downstream of the receptors, BMPs phosphorylate Smad proteins. The so-called BMP-specific proteins include Smad1, Smad5 and Smad8, known as receptor-regulated Smads or R-Smads. R-Smad, in turn, bind to Smad4 and this complex translocates to the nucleus, where it can bind to transcription factors either stimulating or inhibiting gene transcription. In addition, Smad6 and 7 (inhibitory Smads or I-Smads) compete with R-Smad for Smad4 binding, therefore preventing Smad1/5/8 nuclear translocation and regulation of BMP-associated genes. The induction of gene transcription downstream of BMP2 requires connexin (Cx) 43 expression, and deletion of Cx43 from calvaria osteoblastic cells inhibits osteoblast differentiation induced by BMP-2 *in vitro* (Hashida et al., 2014). This effect of Cx43 requires direct cell-to-cell communication and cannot be restored by a Cx43 mutant that lacks the ability to form gap junction channels (see below for further description of connexins).

One of the transcription factors regulated by Smads downstream of BMP action is Runt-related transcription factor 2 (Runx2), which is required for osteoblast differentiation (Komori, 2006). Activation of the BMP/Smad signaling pathway results in the transcription of proteins involved in osteoblast differentiation and functions, such as osteocalcin, collagen alpha-1(I) chain, and alkaline phosphatase. In addition to activating Smads (the canonical BMP signaling pathway), BMPs also activate the non-canonical MAPKKK 7/TGF-β-activated kinase 1 (TAK1)-p38 pathway, independent of Smads (Gomez-Puerto et al., 2018), a pathway required for bone and tooth development (Xu et al., 2008).

In addition to the intracellular regulators, BMP activity is regulated by extracellular antagonists and potentiators (Katagiri and Watabe, 2016). Antagonists include chordin, follistatin, gremlin, and noggin, among others, which sequester BMPs in the extracellular space or block ligand-receptor binding. BMPs potentiators include heparin, heparan-sulfate and dextran-sulfate, which bind to BMPs and potentiate the pro-osteoblastic effect of BMP-2, −4 and −7 (Takada et al., 2003, Irie et al., 2003, Ruppert et al., 1996). Other BMP potentiators have been described, although their role in osteoblastic cells is not known (Katagiri and Watabe, 2016).

The importance of BMP signaling for skeletal development, bone strength, and fracture repair has been demonstrated by human mutations in the ligands, receptors, antagonists, and intracellular signaling molecules (Wu et al., 2016). Further, animal models with both loss-

and gain-of-function mutations have been developed, allowing for a better understanding of the role of the pathway and the consequences of its manipulation for human diseases.

3.2. Wnt-induced signaling

Wnts are a family of secrete glycoproteins originally described in *Drosophila*, as the Wingless (*Wg*) gene that is involved in wing development and in mice, as the *Int-1* gene that is involved in breast cancer development (Nusse and Varmus, 1992, Nusse et al., 1991). Wnts induce intracellular signaling by binding to members of the frizzled (FZD) family of receptors, which comprise 10 members (Schulte, 2015). FZDs are seven transmembrane proteins, with a structure similar to G protein-coupled receptors. In addition, all members of the FZD family contain a cysteine-rich domain in the amino terminus that constitute the ligand-binding site, and a cytoplasmic tail that interacts with intracellular molecules. Wnts also interact with members of the low density lipoprotein receptor protein (LRP) family, which includes LRP4, LRP5 and LRP6. While LRP5 and 6 have been shown to mediate the activation of the Wnt signaling pathway, LRP4 binds to and facilitates the action of the Wnt antagonist sclerostin, the product of the Sost gene (Williams, 2017).

Through its binding to FZD receptors, Wnt ligands can activate four different signaling pathways: the canonical Wnt/β-catenin pathway, the non-canonical planar cell polarity (PCP) pathway, the Wnt-Ca2+, and the protein kinase A pathways (Baron and Kneissel, 2013). It has been shown that members of the Wnt family of proteins can activate one particular pathway in a cell context-dependent manner. Nevertheless, activation of both canonical and non-canonical Wnt signaling result from Wnt proteins binding to FZD-LRP5/6 co-receptor complex, followed by phosphorylation and activation of Disheveled (Dsh).

Activation of canonical Wnt signaling leads to the stabilization of β-catenin, followed by its translocation to the nucleus, where β-catenin binds to the transcription factor LEF and increases the transcription of Wnt target genes. In osteoblastic cells, osteoprotegerin (OPG) is one of the most studied Wnt target gene. OPG is involved in osteoclast differentiation and counteracts the pro-osteoclastogenic effects of receptor activator of NFκB ligand (RANKL), resulting in decreased osteoclast differentiation (see below for more details on osteoclastogenesis). Consistent with this, decreased bone mass induced by targeted deletion of the gene encoding β-catenin in osteoblasts and osteocytes results from enhanced bone resorption due to low OPG levels.

The role of Wnts in bone has been elucidated through understanding the genetic basis of human diseases associated with the pathway, and confirmed through genetic studies in which the different components of the signaling pathways have been modified either in cells of the osteoblastic lineage or in the germline (Baron and Kneissel, 2013). For example, both gain- and loss-of-function mutations have been described for LRP5, leading to high bone mass phenotypes and osteoporosis pseudoglioma, respectively (Williams, 2017). In addition, high bone mass phenotypes have also been described in individuals with loss of function mutations of the LRP4 gene (Leupin et al., 2011). Further, absence of sclerostin due to null mutations, or reduced expression of the protein due to absence of a regulatory region of the

gene result in sclerosteosis or van Buchem's disease, respectively, conditions with high bone mass (Williams, 2017).

In addition, mutations in Wnt10b result in split hand/foot malformation 6 (Kantaputra et al., 2018, Zmuda et al., 2009) and deletion of this Wnt in mice leads to decreased bone marrow mesenchymal progenitors, low bone mass, and reduced expression of osteoblastic differentiation markers (Stevens et al., 2010). Further, osteoblast-derived Wnt16 suppresses osteoclast formation by increasing OPG levels (Moverare-Skrtic et al., 2014, Alam et al., 2016), and SNPs associated with loss of Wnt16 function are linked to low BMD and osteoporotic fractures (Koller et al., 2013).

The associated of certain Wnts with particular FZD receptors lead to the phosphorylation of Dsh, followed by the activation of the PCP pathway, downstream of the small GTPases Rho and Rac, and the MAPK/c-Jun N-terminal kinase (JNK). The PCP pathway is involved in cytoskeletal organization, cell migration, and tissue pattering, and ultimately, in tissue morphogenesis. In bone, PCP is involved in the control of limb shape and dimensions. Thus, activation of PCP downstream of Wnt5a results in the transcription of the gene *Vangl2*, and absence of *Vangl2* leads to deficient limb bud development in mice. In addition, individuals with loss-of-function mutations in the Wnt5a gene or its co-receptor ROR2 exhibit brachydactyly type B and Robinow syndrome, conditions associated with shortening of long bones, and abnormal bone shape, among other abnormalities.

Further studies showed that the non-canonical members of the Wnt family of proteins Wnt4, Wnt5a, Wnt7b, and Wnt11 increase MSCs differentiation into the osteoblastic lineage. For example, Wnt5a stimulates osteogenic differentiation and inhibits adipogenesis in human adipose tissue-derived MSCs through activation of the Rho-associated protein kinase ROCK. In addition, the expression of Wnt5a and its co-receptor ROR2 are increased by mechanical stimulation, leading to RhoA activation, which is required for upregulation of Runx2 and the commitment of precursor cells to the osteogenic lineage induced by mechanical stimulation. As discussed later in this review, Wnt5a also plays a direct role in osteoclast differentiation.

3.2.1. Inhibitors of Wnt Signaling—Activation of the Wnt signaling pathway is controlled by the regulation of expression and secretion of Wnt proteins, as well as by a network of inhibitors that act at different levels of the signaling cascade (Plotkin and Bivi, 2014, Lerner and Ohlsson, 2015). In bone, human mutations and animal experiments have demonstrated the importance of the inhibitors in the regulation of bone cell differentiation and function and, ultimately, bone mass and strength. Further, pharmacologic approaches are being developed to take advantage of the anabolic effect of Wnt signaling in bone, by blocking the Wnt antagonist, leading to increased bone mass (Baron and Gori, 2018).

3.2.1.1. Dickkopf: The Dickkopf (Dkk) proteins are members of a family of cysteine-rich domain containing proteins (Baron and Kneissel, 2013). Dkk1 and Dkk2 inhibit Wnt signaling by binding to LRP5/6 and Kremen (another Wnt inhibitor, see below). The expression of Dkk1, the most studied member of the family, is hormonally regulated, and it is also controlled by growth factors and mechanical stimulation. Dkk1 expression is required for limb morphogenesis and in its absence the head does not develop in Dkk1 knockout mice

(Huang et al., 2018). On the other hand, deletion of one Dkk1 allele results to high bone mass due to increased osteoblast number and activity, whereas Dkk1 overexpression results in reduced osteoblast number and activity and low bone mass. Consistent with an inhibitory role of Dkk1 on osteoblast function, high bone mass-associated LRP5 mutations render LRP5 proteins resistant to Dkk1-mediated inhibition of Wnt signaling. Dkk2 and Dkk4 have also been shown to inhibit canonical Wnt signaling in the presence of Kremen.

3.2.1.2. Kremen: The two members of this family of proteins, Kremen 1 and 2 are co-receptors for Dkk1, enhancing Dkk1-induced Wnt signaling inhibition (Baron and Kneissel, 2013, Plotkin and Bivi, 2014). Kremen 1 is widely expressed whereas Kremen 2 is predominantly expressed in bone cells. Kremen 2-deficient mice exhibit an age-dependent high bone mass phenotype, which can be detected at 2 years of age, and Kremen 1 and 2 double knockout mice exhibit increased Wnt signaling, bone mass, and bone formation. Conversely, transgenic mice overexpressing Kremen 2 exhibit reduced osteoblastic Wnt signaling and differentiation and activity and low bone mass.

3.2.1.3. Sclerostin: The product of the Sost gene, sclerostin, is expressed in osteocytes in bone and was originally described as an inhibitor of BMP due to its homology with known BMP antagonists (Baron and Kneissel, 2013, Plotkin and Bivi, 2014). It is now known that sclerostin binds with higher affinity to LRP5/6 and that LRP4 is required for the inhibition of Wnt signaling by sclerostin. As a consequence of its Wnt inhibitory effects, the absence of the Sost gene, reduced sclerostin levels or prevention of sclerostin association with LRP5/6 results in high bone mass. More recently, it has been shown that sclerostin can affect osteoclast differentiation indirectly, by stimulating RANKL expression in osteocytic cells *in vitro* and RANKL/OPG levels *in vivo* (Tu et al., 2015, Wijenayaka et al., 2011). However, whether this action of sclerostin depends on its binding to LRPs or on the regulation of Wnt signaling pathway, and the intracellular signaling molecules involved in RANKL/OPG regulation in osteoblastic cells remains unknown.

3.2.1.4. Secreted Frizzled-Related Proteins: Secreted FZD-related proteins (sFRPs) are soluble proteins that bind to Wnts and prevent their association with the FZD receptors on the cell surface, inhibiting intracellular Wnt signaling (Baron and Kneissel, 2013, Plotkin and Bivi, 2014). Among the five members of the family of proteins, sFRP-1 was shown to be expressed in bone and to inhibit osteoblast differentiation and induce osteoblast and osteocyte apoptosis. Consistent with this, deletion of *Sfrp1* results in high bone mass whereas its overexpression decreased bone mass in transgenic mice. sFRP4 also inhibits bone formation by preventing osteoblast proliferation, and its overexpression in bone or liver lead to elevated sFRP4 circulating levels and low bone mass. Consistent with a role of the protein in bone, *SFRP4* polymorphisms have been associated with changes in bone mass in the hip and spine in humans.

3.2.1.5. Wise: Wise is another LRP5/6 binding protein that prevents Wnt signaling (Baron and Kneissel, 2013, Plotkin and Bivi, 2014). It has also been shown to associate with LRP4, and mutations of both Wise and LRP4 genes lead to similar abnormal teeth, suggesting that the two molecules interact during tooth development. The absence of Wise results in the

activation of Wnt signaling and the modulation of the fibroblast growth factor (FGF) and sonic hedgehog pathways.

3.3. Insulin-Like Growth Factor

Insulin-like growth factor (IGF) I and II, named based on their structural similarity with insulin, and their insulin-like properties, are produced mainly in the liver and also in bone and adipose tissue (Yakar et al., 2018). The two forms of the ligand share high homology. IGFI is expressed both during embryonic development and after birth, whereas IGFII is expressed in the embryo but is downregulated after birth. IGFs bind to and activate two receptors, IGF-IR and IGF-IIR, and can also bind to the insulin receptor, albeit with low affinity. IGF-IR is a tetramers formed by two extracellular ligand binding α subunits and two transmembrane β subunits, which have tyrosine kinase activity. IGF-IR activation results in the phosphorylation of adaptor insulin receptor substrate (IRS) proteins or of Shc (Bikle, 2008), followed by activation of the growth factor receptor binding protein 2 (Grb2)/Sos/Ras/Raf/MAPK extracellular signal–regulated kinase kinase (MEK), resulting in extracellular signal-regulated kinase ERK activation. In addition, activation of the IGF-IR also results in the activation of PI3K, followed by phosphorylation and activation of Akt, and of the mammalian target of rapamycin (mTOR), eukaryotic translation initiation factor 4E (eIF4E), and p70S6 kinase (S6K). In general, activation of this receptor leads to cell proliferation and survival and has been associated with cancer cell metastatic potential. The IGF-IIR, also known as cation-independent mannose-6-phosphate receptor, has no intrinsic kinase activity and binds to IGF-II with high affinity, inducing IGF-II lysosomal degradation. IGF-IR is expressed in bone during development and in adults, whereas mRNA expression for IGF-IIR has been shown in embryos but not adult rat bones (Bikle et al., 1994). The activity of IGFs is modulated by the IGF binding proteins (IGFBP) 1–6, which regulate IGF bioavailability and can either enhance or reduce IGF effects (Bunn and Fowlkes, 2003). IGFBPs also exert direct effects on cell function, independent of IGFs. IGFs and IGFBPs form a complex with the acid-labile subunit (ALS), which is required for IGF/IGFBP circulation and IGF-I function (Boisclair et al., 2001).

Both IGF-I and II are produced locally by osteoblastic cells and stored in the bone matrix as an inactive form bound to IGFBPs (Kasukawa et al., 2004). During bone resorption by osteoclasts, IGFs are released from the bone matrix and can act as signals to recruit osteoblast precursors to the newly eroded bone surface. Osteoblasts also produce several IGBPs, with a pattern that depends on the stage of osteoblast differentiation. The role of IGFs on osteoblast activity has been extensively studied *in vitro* and *in vivo*, using genetically modified mice in which the levels of the ligands and/or the receptors were manipulated globally or in a cell-specific manner (Yakar et al., 2018). In particular, it has been shown that IGFs induce osteoblast proliferation and differentiation and inhibit apoptosis (Bikle, 2008). The importance of IGF on bone development has been demonstrated by the profound skeletal defects observed in mice lacking IGF-I or IGF-IR, with impaired growth and delayed ossification, and reduced bone mass and osteoblast number and surface. The growth deficiency and delayed ossification observed in IGF-IR-deficient mice are reproduced in mice lacking Akt1/Akt2, a kinase activated by IGFs, demonstrating the importance of this signal transduction pathway for bone growth (Peng,

2003). In osteoblastic cells, IGF-I has been shown to activate the PI3K/PKD/Akt and Ras/Raf/MAPK pathways, both leading to activation of p70S6, which mediate the proliferative and anti-apoptotic actions of IGF-I (Govoni, 2012). A pathway activated by IGF-I via $G_{i\beta\gamma}$ leading to osteoblastic cell proliferation and survival independently of p70SK activation has also been proposed (Grey et al., 2003).

3.4. Parathyroid Hormone-Related Peptide

Parathyroid hormone-related peptide (PTHrP) is a locally produced parathyroid hormone (PTH) analog expressed by osteoblasts and osteocytes (Schluter, 1999). Both proteins share high homology at the first 34 aminoacids and bind to the PTH receptor 1 (PTH1R) expressed in osteoblasts and osteocytes. However, they differ in pattern of expression and exhibit different functions; and while PTHrP deletion is lethal due to defective rib bone formation, PTH null mice show a milder phenotype and survive after birth. PTHrP is required for the commitment of osteoblast precursors to the osteoblastic lineage and for osteoblast maturation, and through actions on osteoblastic cells, on osteoclast differentiation via increases in RANKL expression.

Upon binding to the PTH1R, PTHrP induces the phosphorylation of the receptor and the activation of GTP-binding proteins. In osteoblasts, PTHrP binding to PTH1R results in the activation of the Gsα subunit of the G protein leading to cAMP/PKA signaling. PKA, in turn, phosphorylates and activates transcription factors, including CREB, c-Fos, c-Jun, and the osteoblastic transcription factor Runx2.

PTHrP can also activate of the G_q subunit downstream of the PTH1R. This results in activation of the 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase [phospholipase C (PLC)] and PI3K/diacylglycerol pathways, which increase intracellular $Ca2^+$ and stimulate PKC activity. Further, PTHrP can either activate or inhibit the MAPK ERK1/2 signaling pathway, depending on whether cells are undergoing proliferation or differentiation, respectively.

The activity of PTHrP is regulated by metalloproteinases that cleave the protein generating the $PTHrP_{1–17}$ peptide, which can stimulate calcium signaling but cannot activate cAMP. In addition, a $PTHrP_{12–48}$ proteolytic product regulates the bone marrow niche and acts as a suppressor of osteoclastogenesis. A $PTHrP_{107–139}$ peptide is also found *in vivo* and displays anti-resorptive and pro-osteoblastic properties when administered to animal models that lack sex steroids, have excess glucocorticoids, or have diabetes (Esbrit et al., 2016, Lozano et al., 2011).

Another way of regulating PTHrP signaling is through internalization of the receptor leading to desensitization of the cells to PTHrP (as well as PTH) effects. The internalization occurs upon ligand binding, by the recruitment of the scaffolding proteins β-arrestin-1 and −2. Binding to β-arrestins reduce the affinity of PTH1R for Gsα and suppresses cAMP-mediated responses. Receptors can be later recycled to the cell surface or targeted for degradation.

PTHrP also exert nuclear actions in osteoblastic cells, independent of PTH1R. Thus, PTHrP translocate to the nucleus where it can regulate apoptosis and cell proliferation by altering the pattern of gene expression (Garcia-Martin et al., 2014).

3.5. Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are a family of proteins that exert both paracrine and endocrine functions (Beenken and Mohammadi, 2009). FGFs bind to the extracellular matrix until activated by proteases, when they are released to activate FGF receptors (FGFRs). Four FGFRs (1–4) have been described, all members of the tyrosine kinase receptor family, and FGFR1–3 are expressed in osteoblastic cells (Ornitz and Marie, 2015). Binding of FGFs to the receptors results in the activation of the intracellular signaling molecules PLC-γ1, MAPK, and PI3K/Akt (Yun et al., 2010). In addition to direct actions of FGFs through their receptors, the growth factors affect bone cells indirectly, through the up-regulation of other local factors, such as TGFβ, IGF-I and VEGF (Ornitz and Marie, 2015).

Genetic studies in mice showed differing effects of different FGFR in osteoblastic cells. Thus, FGFR2 deletion in osteochondroprogenitors results in shorter axial and appendicular skeleton and reduced bone formation, whereas FGFR1 deletion in osteoprogenitors and differentiated osteoblasts results in increased bone mass, suggesting that the receptor mediates inhibitory actions on osteoblastic cells (Yu et al., 2003, Jacob et al., 2006).

Regarding FGFR ligands, FGF2 is required for proper osteoblast differentiation and activity, and its deletion results in decreased bone mass in mice (Yu, 2003). The mechanism of action of FGF2 and the consequence of overexpression of the growth factor depend on the molecular weight isoform, with the low molecular weight inducing activation of the WNT/β-catenin signaling pathway and increased bone formation (Xiao et al., 2009), and the high molecular isoform inducing the activation of the FGF23/FGFR/MAPK signaling pathway and leading to reduced bone formation *in vitro* (Xiao et al., 2012). *In vitro* studies demonstrated that the pro-osteoblastogenic effects of FGF2 are potentiated by expression of Cx43 (Hebert and Stains, 2013, Lima et al., 2009, Niger et al., 2012, Niger et al., 2010, Niger et al., 2013) (see below). In addition, FGF2 promotes osteoblast survival through the activation of the PI3K signaling pathway (Park et al., 2009).

Other members of the FGF family of proteins with action on bone cells are FGF18, which is expressed in osteoblastic cells and induces osteogenic differentiation through activation of the FGFR1/2 and the MAPK and PI3K signaling pathways *in vitro* (Hamidouche et al., 2010), and FGF21, which is produced in pancreas, liver, and adipose tissue and decreases osteoblast surface, and bone mass and strength (Charoenphandhu et al., 2017).

Bone, and in particular, mature osteoblasts and osteocytes, are the main source of FGF23, a growth factor that reduces vitamin D levels and regulates phosphate homeostasis through inhibition of renal phosphate reabsorption (Yoshiko et al., 2007). FGF23 synthesis is, in turn, regulated by phosphate levels, vitamin D and PTH (Clinkenbeard and White, 2016). Activation of the FGFR by FGF23 requires the expression of the co-receptor Klotho, expressed in kidney (in the distal convoluted tubule) and the parathyroid gland (Clinkenbeard and White, 2016, Komaba and Lanske, 2018). More recently, it was shown

that Klotho is also expressed in bone and, in particular, in osteoblasts and osteocytes (Rhee et al., 2011). Further studies showed that absence of Klotho from osteocytes results in increased bone mass and osteoblast activity in mice, whereas Klotho overexpression inhibits osteoblastic cell differentiation *in vitro* (Komaba and Lanske, 2018). The mechanism for the actions of FGF23 on osteoblastic cells is not completely understood, but it is believed to be mediated by induction of the expression of the Wnt/β-catenin inhibitor Dkk1, resulting on the inhibition of the bone anabolic Wnt pathway. In addition, FGF23 regulates its own synthesis through Klotho in osteoblastic cells. The effects of FGF23 on osteoblastic cells via FGFR1/Klotho stimulation result in the activation of ERKs, leading to modulation of gene expression.

4. OSTEOCLAST SIGNALING MECHANISMS

Osteoclasts are the predominant bone-degrading cell in the body (Figure 1). The highly motile nature of osteoclasts, combined with their resorptive capacity, can result in extensive degradation of cortical bone, cancellous bone, alveolar bone, and tooth dentin. The molecular analysis of human forms of osteopetrosis caused by defects in osteoclast resorption, and the prevalence of low bone mass osteoporosis especially in the elderly which is due to excessive osteoclast activity, highlight the importance of understanding osteoclast biology for human disease. The catabolic effects of osteoclasts must therefore be tightly controlled, which occurs in part through the regulation of osteoclast formation and cell death, as well as the expression and secretion of bone degrading chemical and biological factors. The birth, life, and death of osteoclasts is regulated by a complex series of signaling molecules and pathways, many of which are known, while others still remain to be determined.

4.1. Osteoclast Morphology and Function

4.1.1. Regulation of Osteoclast Resorption by Proton Pump and Chloride Channel—Upon attachment to bone, osteoclasts change their morphology to become polarized multinucleated cells, which enables them to form two specialized surface domains, known as the sealing zone and the ruffled border membrane. The sealing zone surrounds the ruffled border and provides the machinery to enable attachment to the extracellular matrix. The ruffled border, on the other hand, is a highly convoluted membrane domain through which the hydrolytic enzymes and factors necessary for bone resorption are secreted (Baron et al., 1985, de Vernejoul et al., 1988, Baron et al., 1988, Blair et al., 1989, Vaananen et al., 2000, Bruzzaniti and Baron, 2006).

The ruffled border contains an extensive amount of enfolded membrane, which is formed from the directed fusion of intracellular vesicles with the apical domain of the osteoclasts. The ruffled border membrane provides a large surface area through which bone dissolution and endocytosis of bone degradation products can occur. The degradation products of collagen and other matrix components are endocytosed at the ruffled border, transported through the cell to be degraded or secreted, or are released into the extracellular space during cell migration.

During bone resorption, the osteoclast subcellular lacunar compartment is acidified through the combined actions of the vacuolar ATPase (proton pump) (Scimeca et al., 2000, Wu et al., 2009, Lee et al., 2006, Feng et al., 2009, Yang et al., 2012), in conjunction with chloride ions secreted by the chloride channel CIC-7 (CLCN7) (Kasper et al., 2005, Kornak et al., 2001). Numerous mutations of the vacuolar ATPase or CIC-7 are known, which cause dysfunction in osteoclast resorption, leading to osteopetrosis in mice (Kornak et al., 2001) and humans (Cleiren et al., 2001, Waguespack et al., 2003). The targeted delivery of protons and chloride ions to the osteoclast resorption lacuna lowers the pH and dissolves hydroxyapatite while a mixture of proteases, including matrix metalloproteases such as MMP9 (Kim et al., 2016, Sundaram et al., 2007) and MMP14 (Sato et al., 1997) as well as tartrate-resistant acid phosphatase (TRAP) (Marks and Grolman, 1987, Allen et al., 1989) and cathepsin K (Troen, 2004, Gelb et al., 1996, Lazner et al., 1999, Gowen et al., 1999), a cysteine protease expressed predominately in osteoclasts, digest the organic bone matrix.

The extrusion of protons by the vATPase and chloride ions through the CIC-7 alters intracellular membrane polarity. Therefore, proton generation in mature osteoclasts is controlled by carbonic anhydrase II, an enzyme that catalyzes the interconversion of carbon dioxide and water to form protons and carbonic acid. In addition, electroneutrality within the osteoclast is maintained by the actions of CIC-7. These channels function as Cl-/H+ antiporters such that for every two chloride ions that are released into the resorption lacuna, one hydrogen ion flows in. The actions of CIC-7 also require OSTM1 (osteopetrosis-associated transmembrane protein 1) which is a transmembrane protein also localized to lysosomes which is thought to function as a β subunit of CIC-7, controlling its intracellular localization and protein stability (Chalhoub et al., 2003, Lange et al., 2006, Maranda et al., 2008, Pangrazio et al., 2006).

4.1.2. Small GTP-Binding Proteins—Several small GTP-binding proteins are known to be expressed in osteoclasts and play a role in targeting the acid transporting vesicles to the ruffled border membrane (Mulari et al., 2003b, Mulari et al., 2003a, Sun et al., 2005, Itzstein et al., 2011). For example, Rab5c is known to be associated with early recycling endosomes, while Rab11b is associated with cytosolic (perinuclear) recycling compartments and is thought to regulate turnover of the ruffled border membrane, and osteoclast motility. In addition, the GTP-binding proteins, Rab7 and Rab9, which are known to be associated with the late endosomes in other cells, were found to localize at the ruffled border membrane, suggesting that this specialized plasma membrane domain may behave similar to a late endosomal compartment. Several lysosomal proteins are also expressed at the ruffled border membrane and in the resorption lacuna, suggesting a lysosomal origin for these vesicles (Ferron et al., 2013, Lacombe et al., 2013). Indeed, the resorption lacuna acts much like an extracellular lysosome, providing the acidic environment as well as the proteases required for the degradation of the organic matrix of bone, mostly composed of type I collagen.

4.1.3. Osteoclast Attachment and Migration—Osteoclast attachment is mediated though integrins, which bind to arginyl-glycyl-aspartic acid (RGD) peptides at eroded bone surfaces (Shankar et al., 1995, Fisher et al., 1993, Horton et al., 1991, Helfrich et al., 1992). The integrins, as well as host of other cytoskeletal proteins, and filamentous actin, are

localized to the sealing zone of osteoclasts. Osteoclasts resorption is linked with their motility, which is in part facilitated through the rapid polymerization and depolymerization of actin, as well as the assembly/disassembly of signaling molecules at adhesion “hot spots” in the sealing zone known as podosomes (Jurdic et al., 2006). Podosomes are not just found in osteoclasts, but also in highly migratory cells such as macrophages and v-src transformed cancer cells (Spinardi et al., 2004, Linder and Aepfelbacher, 2003, Destaing et al., 2003). Structurally, podosomes contain a central actin core surrounded by actin regulatory proteins, integrins, and the integrin-associated proteins such as talin, cortactin, dynamin (McNiven et al., 2004, Ochoa et al., 2000). In addition, they contain a large number of signaling molecules such as Src, Pyk2, talin, cortactin, and others. Unlike focal adhesions, which are relatively more stable structures, podosome clusters assemble and disassemble within minutes in a process involving the rapid polymerization and depolymerization of the central actin core (McNiven et al., 2004, Ochoa et al., 2000). Thus, podosome turnover and targeted integrin engagement/disengagement in the sealing zone enables osteoclasts to rapidly migrate and, in the process, digest significant amounts of bone during their short life spans. Indeed, in studies published as far back as 1984, it was reported that rabbit osteoclasts in culture display an average resorption rate of 165 $\mu m^3/h$ (Ali et al., 1984, Boyde et al., 1984).

4.2. Osteoclast Differentiation

Osteoclasts are derived from the hematopoietic, myeloid lineage. Many of the extracellular signals that drive osteoclast proliferation, differentiation, and fusion are secreted by the mesenchymal-derived osteoblasts and osteocytes. The osteoclast differentiation process can be recapitulated *in vitro* by the addition of two primary cytokines, macrophage colony-stimulating factor (M-CSF) and RANKL (Figure 3) (Kong et al., 1999, Wiktor-Jedrzejczak et al., 1990), which are secreted by osteoblasts and osteocytes, as discussed previously in this review.

4.2.1. M-CSF/c-FMS—The critical role of M-CSF is demonstrated from deletions/mutations studies in mice (op/op) and rats (tl/tl) which have a point mutation in the *csf1* gene and express non-functional M-CSF protein (Felix et al., 1990, Marks et al., 1992). As a result, osteoclasts are absent in these mice, leading to severe osteopetrosis. The predominant role of M-CSF is in supporting the proliferation and survival of osteoclasts. Binding of M-CSF to its receptor c-Fms (or CSFR-1R) induces phosphorylation at key tyrosine sites in the cytoplasmic tail of c-Fms, which recruits the tyrosine kinase c-Src (Figure 3). The c-Fms/c-Src protein complex then recruits the phosphatidylinositol 3-kinase (PI3K) and the E3 ubiquitin ligase, c-Cbl, in turn leading to the activation of the Akt pathway, and subsequently to the Grb2-mediated activation of ERK (Horne et al., 2005, Yokouchi et al., 2001, Sanjay et al., 2001, Lee and States, 2000, Xiong et al., 2011). Therefore, through a complex series of steps, M-CSF binding to c-Fms, increases osteoclast precursor proliferation and survival, ultimately through the ERK and PI3K/Akt pathways.

4.2.2. Signaling by the RANK Receptor—RANKL (also known as OPGL, ODF and TRANCE) binds to its cognate receptor, RANK (Figure 3). As with c-Fms, genetic mouse models have demonstrated the critical role of the RANKL-RANK system in osteoclast formation, and mice deficient in either the ligand or the receptor develop osteopetrosis

(Walsh and Choi, 2003, Kong et al., 1999, Hanada et al., 2010, Odgren et al., 2003). The binding of RANKL to its receptor, RANK, on osteoclasts induces the association of RANK with the TRAF family members, including TRAF2 and TRAF6. Among these, TRAF6, has been most strongly associated with osteoclast formation and function and mice lacking TRAF6 develop osteoclast-deficient osteopetrosis (Naito et al., 1999, Lomaga et al., 1999). Downstream effectors of the RANK-TRAF6 complex in osteoclasts, include NF-κB, Akt, and Nuclear Factor of Activated T-cells cytoplasmic 1 (NFATc1). Further, the activation of PI3K/Akt may be dependent on Src kinase activity, since in the absence of active c-Src, RANKL-mediated Akt activation is prevented and PI3K inhibitors block osteoclast formation. In addition to activating the PI3K/Akt pathway, TRAF6 binding to RANK, recruits TGF-β-activated kinase (TAK) 1 and the TAK-1-binding protein (TAB) 2, and this protein complex activates the MAPK pathways including ERK, JNK, and p38, promoting pre-osteoclast proliferation (Moon et al., 2012, Sugatani et al., 2003, Wong et al., 1999).

In contrast to the pro-osteoclastic effects of RANK-RANKL activation, an alternative receptor for RANKL in osteoclasts has recently been described which leads to the inhibition of osteoclast development. The leucine-rich repeat-containing G-protein coupled receptor 4 (LGR4, also known as GPR48) is a suppressor of osteoclast formation (Styrkarsdottir et al., 2013, Yi et al., 2013, Luo et al., 2016, Zhu et al., 2016, Jin and Yang, 2016, Zhou et al., 2017). RANKL binding to LGR4 activates Gαq, calcium signaling, and GSK3-β which lead to the inhibition of NFATc1 in the nucleus, blocking osteoclast differentiation. In support of the critical role of LRG4 in osteoclastogenesis, deletion of LRG4 in all tissues or specifically in monocytes leads to osteoporosis in mice, due to increases in osteoclast number and activity. Further, it was demonstrated that the soluble extracellular domain of LGR4 can bind RANKL, inhibiting osteoclast differentiation and increasing bone mass in several mouse models. Therefore, LGR4 acts as a second receptor for RANKL that negatively regulates osteoclast formation and activity.

4.2.3. Osteoclast Transcription Factors—Signaling through the c-Fms and RANK receptors leads to the activation of important osteoclasts transcription factors, including microphthalmia-induced transcription factor (MITF) and NFATc1 (Meadows et al., 2007). These transcription factors drive the expression of several genes necessary for osteoclast resorption. For example, in response to RANKL-RANK binding, MITF upregulates TRAP and cathepsin K, which are required for the ability of mature osteoclasts to degrade the bone matrix. MITF is also known to regulate the expression of ClC-7 and Ostm1.

RANKL also activates NF-κB through two distinct pathways, known as the classical pathway or the alternative pathways. NF-κB consists of five members, including p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). Activation of NF-κB can have both positive and negative effects on osteoclast activity (Boyce et al., 2015). While TRAF6 activates only the classical NF-κB pathway, TRAF2 and TRAF5 activate both pathways (Hauer et al., 2005, Yi et al., 2014, Mizukami et al., 2002). When activated by RANKL, NF-κB translocates to the nucleus and binds its target genes, leading to the upregulation of c-Fos. Subsequently, c-Fos binds to NFATc1. Thus, NF-kB positively regulates osteoclast differentiation and function by activating c-Fos and NFATc1, as well as by inhibiting specific NFATc1 repressor proteins. Conversely, NF-kB can inhibit osteoclast

formation and function through TRAF3 and P110, and the suppressors of c-Fos/NFATc1 signaling, IRF8, and RBP-J. NFATc1 is also regulated by calcium/calmodulin and it is thought that RANKL-mediated phosphorylation of specialized motifs known as immunoreceptor tyrosine-based activation motifs (ITAMs). Phosphorylation of ITAMs result in the activation of Syk and PLCγ, which mobilizes intracellular calcium and, in turn activates calcineurin, a calmodulin-dependent phosphatase that directly dephosphorylates serine residues in NFATc1. Two specific tyrosine kinases, BTK and Tec, may provide the molecular link between ITAM and RANK receptor signaling leading to osteoclastogenesis (Shinohara et al., 2008, Pitcher and van Oers, 2003).

It is important to note that osteoclast differentiation can also be stimulated independently of RANKL, through tumor necrosis factor alpha (TNFα), and M-CSF is a common factor required for both RANKL and TNFα-induced osteoclast differentiation (Kobayashi et al., 2000). Osteoclast differentiation induced by TNFα occurs via the TNF receptor (TNFR) 1 and 2 which are expressed on osteoclast precursors. TNFR1 and TNFR2 also use TRAF2 as a common signal transducer, through which they promote NFATc1 activation and osteoclast differentiation (Kanazawa and Kudo, 2005). However, osteoclasts induced by TNFα formed resorption pits only in the presence of interleukin 1 alpha (IL-1α), and therefore play an important role in bone resorption associated with inflammatory bone diseases.

4.2.4. Osteoclast Precursor Fusion Proteins—The formation of a mature multinucleated osteoclasts requires the fusion of mononuclear precursor cells. Two signaling proteins have been identified which regulate osteoclast fusion and multinucleation, namely DC-STAMP (dendritic-cell specific transmembrane protein) and OC-STAMP (osteoclast stimulatory transmembrane protein) (Kukita et al., 2004, Vignery, 2005, Yagi et al., 2005, Miyamoto, 2006, Yagi et al., 2007, Yang et al., 2008, Mensah et al., 2010). DC-STAMP is expressed as a dimer on the cell surface of both human and murine osteoclast progenitors. It was discovered that mice lacking DC-STAMP form mononuclear osteoclast precursors that express TRAP, a key enzyme required for bone resorption, but these mononuclear precursors do not fuse and the DC-STAMP-deficient mice exhibit defects in osteoclast number and activity, leading to osteosclerosis. Conversely, DC-STAMP overexpression increases the formation of mature multinucleated osteoclasts. Although RANKL does not directly bind DC-STAMP it can modulate the expression of DC-STAMP in osteoclast precursors. In recent studies, it was reported that DC-STAMP contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail, and that DC-STAMP regulates osteoclast differentiation through the NFACTc1 signaling (Chiu et al., 2017).

The second osteoclast “fusogenic” protein, OC-STAMP, is also induced by exposure to RANKL (Kim et al., 2011, Yang et al., 2008). Unlike DC-STAMP, OC-STAMP appears to only be expressed in osteoclast lineage cells. In murine osteoclast progenitors, inhibition of OC-STAMP activity through the use of a neutralizing antibody or siRNA-mediated knockdown results in a marked, reduction in the number of mature multinucleated osteoclasts, whereas overexpression of OC-STAMP significantly increases the number of mature osteoclasts. Similar to mice lacking DC-STAMP, mice in which OC-STAMP is blocked contain TRAP-positive mononuclear cells, but few mature multinucleated osteoclasts (Kim et al., 2011, Yang et al., 2008).

4.3. Osteoclast Survival and Apoptosis

To prevent dysregulated bone resorption, osteoclast number is regulated at the level of cell differentiation and by limiting osteoclast life span via programed cell death, or apoptosis. Osteoclast survival is controlled by the same factors that are required for osteoclast activity, including the Ras-ERK pathway, and the PI3K/Akt signaling pathways. The mTOR protein is a target of PI3K activity and activation of this kinase is required for the anti-apoptotic actions of M-CSF, RANKL and TNFα in osteoclasts. Activation of the small GTPase Rac1, and the upstream effector, Vav3, are also involved in M-CSF induced cell survival.

The shortening of the osteoclast life span controls the extent and depth of osteoclast resorption activity. Osteoclast apoptosis is accelerated in the absence of supporting osteoblasts or bone marrow cells, and in conditions leading to low levels of RANKL, M-CSF or IL-1. Detachment of osteoclasts also induces apoptosis, presumably because integrins become disengaged from RGD peptides in the bone matrix.

The ability of osteoclasts to sense calcium in the bone microenvironment occurs through the calcium sensing receptor, CaSR. Activation of the CaSR in osteoclasts in response to calcium induces the PLC-mediated remodeling of their cytoskeleton, leading to decreased osteoclast bone resorbing activity (Mentaverri et al., 2006, Bennett et al., 2001). The PI3K/Akt pathway is also implicated in the response of osteoclasts to extracellular calcium (Bennett et al., 2001, Boudot et al., 2010). Thus, at high concentration of calcium, which mimics the microenvironment of demineralized bone, the activated CaSR drives osteoclast apoptosis (Lorget et al., 2000). Interestingly, at low concentrations of calcium in culture, osteoblasts produce more RANKL, thus driving osteoclastogenesis (Takeyama et al., 2000).

Currently, the majority of drug agents available to protect against bone loss are the anti-resorptive therapies (Stepan et al., 2003). These anti-osteoclastic therapies include estrogen and the selective estrogen receptor modulator (SERM) raloxifene, which both decrease osteoclast life span and promote apoptosis. Estrogens act via a complex signaling mechanism to inhibit the activation of T cells, decreasing their secretion of RANKL and TNFα. Estrogen also inhibits osteoclast differentiation and decreases life span, by regulating the expression of Fas ligand (FasL) in osteoblasts (Krum et al., 2008), which binds the death receptor Fas in osteoclasts (Nakamura et al., 2007a, Nakamura et al., 2007b), accelerating osteoclast cell death. Finally, the anti-resorptive amino-bisphosphonates also decrease bone remodeling by decreasing osteoclast activity and by inducing osteoclast apoptosis (Dunford et al., 2006, Tella and Gallagher, 2014, Tsubaki et al., 2014).

4.3.1. Bcl-2 Proteins and Osteoclast Apoptosis—Apoptosis occurs through either the extrinsic (death receptor) pathway or the intrinsic (mitochondria) pathway (Hengartner, 2000). In the extrinsic pathway, activation of a TNFR induces apoptosis via the activation of caspase 8, an aspartate-specific cysteine protease. However, in the intrinsic pathway, cytochrome c release from the mitochondria is regulated by different Bcl-2 family members which are either anti-apoptotic or pro-apoptotic in action. For example, in mature osteoclasts, cytokine withdrawal leads to reduced expression of anti-apoptotic Bcl-2 and rapid apoptosis (Tanaka et al., 2010). Removal of RANKL signaling also leads to the activation of Bid-induced and caspase-3 induced osteoclast apoptosis (Vaira et al., 2008). M-

CSF withdrawal also decreases Bcl-2 through inactivation of the transcription factor, MITF (Tanaka et al., 2010). Osteoclast apoptosis is also regulated by increased expression of the pro-apoptotic protein, Bim, a BH3-domain-only member of the Bcl-2 family proteins (Wakeyama et al., 2007). In the absence of Bim, mice have decreased osteoclast activity, despite increased cell survival (Tanaka et al., 2010). Bim expression, and therefore apoptosis, is kept in check through the c-Cbl-mediated ubiquitin/protease degradation pathway that is regulated by caspase-3, such that in the absence of caspase-3, the degradation of Bim is suppressed. The expression of Bim is also down-regulated by IL-3 signaling through the Raf/ERK and/or PI3K/mTOR pathways. In contrast, upregulation of the anti-apoptotic protein, Bcl-$X_{L,}$ which decreases Src, and inhibits the cleavage of procaspase-9, initiating osteoclast apoptosis.

4.3.2. FasL-Induced Osteoclast Apoptosis—Osteoclast apoptosis is also believed to be regulated in part through the osteoblast-secreted factor FasL (or CD95L) (Kovacic et al., 2010). FasL is a membrane-bound protein belonging to the TNF family proteins. FasL expression in osteoblasts is regulated by estrogens, and MMP3-induced FASL cleavage in osteoblasts leads to the secretion of FasL, and subsequently to the osteoblast-mediate apoptosis of osteoclasts (Garcia et al., 2013, Krum et al., 2008). FasL binds the prototypical TNF family death receptor, Fas (FS-7-associated surface antigen; CD95; APO1; TNFR superfamily member 6, TNFRSF6) (Wu et al., 2003). Fas, is widely expressed in numerous cells, including macrophages and osteoclast progenitors. After Fas activation the adaptor protein FADD (Fas associated death domain) binds Fas, which leads to the recruitment of procaspase 8 and 10, forming a death-inducing signaling complex (DISC). The DISC complex then activates caspase 8, and consequently caspase 3 in the cytosol, promoting apoptosis. Although Fas is the main receptor of FasL, the decoy receptor 3 (DcR3), another member of the TNFR superfamily, also acts as a decoy receptor for FasL (Sheikh and Fornace, 2000, Yang et al., 2004). DcR3 is a secreted protein and competitively binds and inhibits several TNF family proteins, not just FasL. It was reported that DcR3 acts as a ligand to induce the differentiation of macrophages into osteoclasts. DcR3 also suppresses RANKL-induced osteoclast formation via the NF-kB and NFATc1 pathway (Cheng et al., 2013), thus functioning as a regulator of osteoclast apoptosis.

4.4. Osteoblast to Osteoclast Signaling

In addition to the well-known pro-osteoclastic factors, such as RANKL, M-CSF and Il-1, osteoblasts secrete factors that decrease osteoclast formation and activity. It has long been established that RANKL-mediated osteoclast formation is opposed by OPG, which is expressed by osteoblasts and osteocytes. OPG acts a soluble decoy receptor for RANKL, effectively reducing the local level of RANKL, and consequently, decreasing osteoclast differentiation and cell number. Moreover, in the presence of OPG, increased rates of apoptosis of osteoclasts and osteoclast precursors has been observed which is due to activation of the classic FasL/Fas apoptosis pathway (Wang et al., 2015, Kovacic et al., 2007).

As discussed previously in this review, osteoblasts produce several Wnt family proteins. Wnt5a binds to two different receptor complexes in osteoclast precursors. Wnt5a binds to

the FZD and LRP5/6 signaling complex and activates β-catenin in osteoclasts to promote osteoclastogenesis (Yamashita et al., 2012, Glass et al., 2005). On the other hand, Wnt5a binding to FZD and Ror2 (receptor tyrosine kinase-like orphan receptor 2) upregulates RANK expression in osteoclasts, which increases RANKL-mediated osteoclast formation (Maeda et al., 2012).

Osteoblast to osteoclast signaling also occurs through the semaphorins family proteins. Semaphorin 3A (Sema3A) binds to a specific receptor complex on osteoclast precursors that is composed of neurophilin-1 (Nrp1) and plexin-A1, and activation of this complex inhibits osteoclast differentiation (Hayashi et al., 2012, Li et al., 2017). It has also been shown that another semaphorin, Sema6C/6D, binds to osteoclast precursor to stimulate ITAMs and DNAX-activating protein 12 (DAP12), which enhances RANK signaling and osteoclastogenesis (Takegahara et al., 2006, Kang et al., 2018, Koga et al., 2004). Of note, mice lacking the ITAM adaptor proteins, FcRγ (Fc receptor common gamma subunit) and DAP12 (DNAX-activating protein 12), exhibit defective osteoclast differentiation, leading to severe osteopetrosis. In osteoclast precursors, the FcRγ and DAP12 proteins can also activate calcium signaling through PLCγ which is also important for regulating osteoclast survival (Bennett et al., 2001).

5. REGULATION OF BONE CELL DIFFERENTIATION AND FUNCTION THROUGH CELL-TO-CELL CONTACT

5.1. Notch Signaling

The Notch signaling pathway is activated by direct contact of a cell expressing the Notch ligands Delta-like proteins (Delta1, 3 and 4) and Jagged (1 and 2) and a cell expressing the receptors known as Notch proteins (Notch 1–4) (Artavanis-Tsakonas et al., 1995). Both ligands and receptors are transmembrane protein and their binding leads to activation of intracellular signaling in the cell expressing the receptor (unidirectional). Osteoblasts and osteocytes (as well as osteoclasts) express both component of the Notch signaling system (Canalis, 2018b). As with other molecules affecting osteoblastic cell differentiation and function, the relevance of Notch signaling for osteoblasts and osteocytes biology was put in evidence by the establishment of the link of human mutations with bone diseases (Canalis, 2018a, Zanotti and Canalis, 2012).

Upon ligand-receptor binding, the canonical Notch signaling pathway is activated, resulting in the proteolytic cleavage of the Notch receptor. This cleavage occurs in two steps, the first one as a result of the activity of the metalloproteinase ADAM17/TACE, which removes the extracellular domain of the receptor. The second step is carried out by the γ-secretase complex that is formed by presenilin-1 and −2, nicastrin, PEN-2, and Aph-1, resulting in the release of the Notch intracellular domain or NICD. The NICD then translocates to the nucleus where it forms a complex with the transcription factors recombining binding protein suppressor of hairless (RBP-Jκ) and mastermind-like (MAML). This trimeric complex recruits co-activators and promotes the transcription of Notch target genes. As for BMP and Wnt signaling, a non-canonical Notch signaling pathway independent of RBP-Jκ has been described, although its role in bone cell is not known (Canalis, 2018b).

Activation of Notch signaling has profound effects on osteoblastogenesis, which depend on the stage of maturation of the cells of the mesenchymal/osteoblastic lineage. Thus, transgenic mice expressing the NICD (which acts as a constitutive active Notch receptor) in osteoblast precursors exhibit high bone mass and increased osteoblast number and proliferation, but impaired osteoblast maturation (Engin et al., 2008, Canalis et al., 2013b). As a results of this altered osteoblastogenesis, immature cells accumulate and produce disorganized, woven bone with accumulation of non-mineralized bone (osteoid). Further, NICD overexpression earlier in the osteoblast differentiation pathway results in reduced bone mass, associated with reduced osteoblast differentiation (Zanotti et al., 2008). On the other hand, NICD overexpression in osteocytes results in increased cancellous bone mass and cortical thickness associated with decreased osteoclast number and bone formation in cancellous bone and no change in osteoclasts but increased osteoblasts in cortical bone (Canalis et al., 2013a). The cellular changes are associated with a decrease in the RANKL/OPG ratio, which favors inhibition of osteoclast differentiation, and a decrease in the Wnt antagonist sclerostin and the consequent increase in Wnt/β-catenin signaling, which favors osteoblast differentiation. These pieces of evidence highlight the cross-talk between different signaling pathways in bone cells. Further *in vitro* studies showed that the Notch/Wnt signaling cross-talk is also involved in the osteoblast-osteocyte transition (Shao et al., 2018). However, in this case it was shown that Notch activation inhibits Wnt/β-catenin signaling, which in turn results in the terminal differentiation of osteocytes. Conversely, activation of Wnt/β-catenin in osteocytes results in activation of Notch signaling (Tu et al., 2015).

Further studies show that double deletion of presenilin-1 and −2 (the enzymes that participate in the release of NICD) or of the Notch receptors-1 and −2 from MSCs in mice results in high bone mass (Hilton et al., 2008). However, presenilin1/2-deficient mice undergo accelerated age-dependent bone loss by a combination of decreased bone formation and increased resorption. The increased resorption occurs indirectly, through increased RANKL/OPG ratio in cells of the osteoblastic lineage.

On the other hand, removal of presenilin-1 and −2 in mature osteoblasts results in decreased bone mass (Engin et al., 2008). However, the phenotype was not due to decreased osteoblast differentiation/activity, as bone formation parameters did not differ between knockout and control mice. Instead, osteoclast number/surface and eroded surface were increased in the mice lacking the presinilins indicating an indirect effect of Notch signaling in osteoblastic cells on osteoclastogenesis.

Osteocytic Notch1/2 deletion results in increased bone mass in both cancellous and cortical bone compartments, due to mechanisms that are both sex- and age-dependent (Canalis et al., 2013a). In addition, the same authors showed that conditional overexpression of Notch ICD in osteocytes results in inhibition of bone formation and resorption, leading to higher bone mass in a low remodeling state (Canalis et al., 2013a, Canalis et al., 2013b).

In addition to the indirect effect on osteoclasts through the control of the RANKL/OPG system, modulation of Notch signaling in osteoclast precursors directly regulates osteoclastogenesis (Canalis, 2018b). Studies have shown that deletion of the canonical

Notch signaling transcription factor RBP-Jk in osteoclast precursors enhances osteoclast differentiation. However, the effect of activation of Notch in these precursors depends on the receptor and while Notch1 inhibits osteoclastogenesis, Notch2 activates it. The effect of Notch2 is associated with the interaction of NICD2 (the fragment of the Notch2 released upon receptor-ligand interaction) with NF-κB, resulting in NFATc1 transcription and osteoclast differentiation. In addition, a study showed that *Notch1* or *Notch3* deletion in bone marrow macrophages enhanced osteoclast differentiation and overexpression of the Notch ligand Jagged1 inhibited osteoclast formation *in vitro* (Bai et al., 2008). On the other hand, another study showed that silencing of Notch2, but not Notch1, inhibits osteoclastogenesis (Fukushima et al., 2008). The reason for the discrepancy between the two studies is not clear, although it is possible that the difference stems from the diverse methods used to remove Notch expression (silencing versus Cre-LoxP system/*in vivo* global gene deletion).

5.2. Ephrin-Ephrin Receptor System

The Ephrin-Ephrin receptor (Eph receptor) system mediates direct cell-to-cell communication through the interaction of the transmembrane proteins Ephrin with the Eph receptor (Cayuso et al., 2015). Unlike the unidirectional Notch signaling, signaling cascades are activated in cells expressing both Ephrin and Eph receptor, constituting a bidirectional signaling system. Two families of Ephrins have been described, ephrinA1–6 and ephrinB1–3 proteins, which bind to EphA1–10 and EphB1–6 receptors, respectively (Committee, 1997). Ephrin ligands are bound to the cell membranes through a glycosylphosphatidylinositol tail (ephrinA) or through a transmembrane domain (ephrinB). The receptors contain intracellular tyrosine kinase domains, which became activated upon ligand binding. The expression of Ephrins and Eph receptors has been demonstrated in osteoblasts and osteoclasts, with osteoblasts expressing ephrinA and B ligands and EphA and EphB receptors, and osteoclasts expressing only ephrinsA2, B1, and B2, but not EphB or EphA1, EphA2, or EphA4 receptors (Zhao et al., 2006). Osteocytes also express ephrinB2 and EphB4 (Wang et al., 2014, Sims and Vrahnas, 2014) and ephrinB1 and B2 (Sims and Martin, 2014). Activation of the Ephrin/Eph receptor system have been proposed to regulate the coupling of bone formation and resorption by triggering signaling in both cells expressing Ephrins and Eph receptors (Lindsey et al., 2018). In particular, *in vitro* studies showed that osteoclast ephrinB2 binds to osteoblast EphB4 resulting in stimulation of osteoblast differentiation by activation of genes such as Runx2 (forward signaling) and inhibition of osteoclast differentiation through decreased expression of the osteoclastic transcription factors c-Fos and NFATc1 (reverse signaling) (Zhao et al., 2006). However, while the existence of this osteoblast-osteoclast interaction has been demonstrated *in vitro*, it is not clear whether it would exist *in vivo*, because osteoblasts and osteoclasts are not normally located in close proximity on the bone surface to allow for direct cell-to-cell contact (Sims and Martin, 2014). On the other hand, a more likely scenario has been proposed, in which ephrinB2/EphB4 is required for osteoblast differentiation and regulates osteoblast-osteoclast precursor interactions. Thus, blockade of ephrinB2/EphB4 interaction results in decreased osteoblast differentiation in the presence of PTH, and in increased osteoclast differentiation *in vitro* and *in vivo*. In addition to this interaction between two different cell types (heterotypic), the role of Eprhin/Eph interaction in osteoblastic cells (homotypic) was shown in mice lacking

ephrinB1, which exhibit decreased osteoblast differentiation and activity (Tonna and Sims, 2014). This phenotype results from the lack of interaction between ephrinB1 and EphB2 in osteoblastic cells, with reduced expression of the transcription factor osterix and of alkaline phosphatase. Conversely, stimulation of ephrinB1 reverse signaling by EphB2 results in increased osterix expression and alkaline phosphatase activity. Similarly, homotypic eprhinB2/EphB4 interaction is required for late stage osteoblast differentiation and blockade of this interaction results in the accumulation of osteoblasts and osteoid, but decreased mineralization. In addition, blockade of eprhinB2/EphB4 interaction leads to increased RANKL expression and osteoclast number in PTH-treated mice.

5.3. Connexins and Intracellular Signaling

Connexins are four transmembrane-spanning proteins that associate in hexamers known as connexons or hemichannels (Beyer et al., 1990). Hemichannels present in neighboring cells align to form gap junction channels that allow the communication between the two cells. Hemichannels can also be found in unopposed cell membranes, where they mediate the interaction of the cell with the extracellular medium (Goodenough and Paul, 2003). In bone cells, hemichannels are involved in the transduction of mechanical, pharmacologic and hormonal stimuli into intracellular signaling (Plotkin and Stains, 2015, Plotkin et al., 2017). In addition to their classic function as channel forming proteins, connexins are also mediators of intracellular signaling, mainly through interaction of the cytoplasmic connexin C-terminus region with structural proteins and enzymes, such as kinases and phosphatases (Leithe et al., 2018). In osteoblastic cells, the interactions between connexins and intracellular signaling molecules is required for prostaglandin release and anti-apoptosis induced by mechanical stimulation, differentiation induced by fibroblast growth factor 2, and cell survival induced by bisphosphonates and parathyroid hormone (Plotkin and Stains, 2015).

The expression and function of connexins in osteoblasts and osteocytes has been extensively studied (Plotkin and Stains, 2015). In particular, Cx43, Cx37, Cx45, and Cx46 are expressed in osteoblastic cells, being Cx43 the most highly expressed and best studied connexin in bone. *In vitro* and *in vivo* studies showed that Cx43 deletion reduces osteoblastogenesis whereas its overexpression leads to increased osteoblast differentiation *in vitro*. Thus, overexpression of Cx43 in osteoblastic cells increases ERK signaling through a mechanism that requires direct cell-to-cell contact and intercellular communication (Stains and Civitelli, 2005, Niger et al., 2012), whereas expression of Cx45, which acts has partial dominant negative effects on Cx43 actions, reduces ERK and Akt activation in Cx43-expressing cells (Stains and Civitelli, 2005). Activation of ERK/PI3K pathways by Cx43 results in increased Sp1 phosphorylation and its recruitment to the promoter of osteoblastic genes such as osteocalcin and osterix, increasing their expression and osteoblast differentiation (Stains and Civitelli, 2005, Niger et al., 2011, Stains et al., 2003).

Further studies demonstrated that increased Cx43 levels in osteoblastic cells *in vitro* enhances the activation of protein kinase C delta (PKCδ) induced by FGF2 (Lima et al., 2009, Niger et al., 2012), through direct binding of PKCδ with Cx43 C-terminal domain (Niger et al., 2010, Hebert and Stains, 2013). In addition, Cx43 increases the transcriptional

activity of the master regulator of osteoblastogenesis Runx2 through activation of inositol polyphosphate/PKCδ signaling in osteoblastic cells (Niger et al., 2013, Niger et al., 2012, Lima et al., 2009).

In addition to its role on osteoblast differentiation and FGF2 actions, Cx43 also mediate the anti-apoptotic effects of bisphosphonates and PTH (Plotkin and Bellido, 2013). In the case of bisphosphonates, addition of the drug to cultured osteocytic cells results in opening of Cx43 hemichannel, and the activation of a signaling cascade that involves the formation of a complex comprising β-arrestin, Scr, MEK and ERKs (Plotkin et al., 2002, Plotkin et al., 2005). This leads to the phosphorylation of the cytoplasmic ERK target p90RSK, which in turn phosphorylates C/EBPβ and BAD, resulting in cell survival. This anti-apoptotic signaling pathway does not required nuclear actions of ERKs or ERK-mediated gene transcription and, instead, it is blocked by forced ERK nuclear accumulation.

Cx43 expression is also required for the anti-apoptotic effect of PTH on osteoblastic cells *in vitro* (Plotkin and Bellido, 2013). As with bisphosphonates, hemichannel but not gap junction channel activity are required for the effect of the hormone. Cx43 associates with β-arrestin through the phosphorylated serine 368 in the C-terminus domain of the connexin (Bivi et al., 2011). Cx43-β-arrestin association release the PTH1R from its binding to β-arrestin, allowing for cAMP accumulation, protein kinase A (PKA) activation and downstream gene transcription.

Cx43 also mediates the activation of intracellular signaling induced by mechanical stimulation in osteocytic cells (Plotkin and Bellido, 2013). In this case, *in vitro* studies showed that Cx43 C-terminus domain interacts with α5β1 integrin, leading to opening of Cx43 hemichannels in osteocytes subjected to fluid flow shear stress (Batra et al., 2012, Batra et al., 2014). Opened Cx43 hemichannels mediate the release of autocrine/paracrine factors such as ATP and prostaglandin E2 (PGE2) (Cherian et al., 2005, Genetos et al., 2007, Siller-Jackson et al., 2008). PGE2, in turn has been shown to mediate the survival effect of mechanical stimulation in osteocytes (Kitase et al., 2010). The protective effect of the prostaglandin is mediated by its interaction with EP2 and EP4 receptors, resulting in the activation of the cAMP/PKA and the Wnt/β-catenin pathways.

Reduced Cx43 levels in osteoblastic cells indirectly affects osteoclast differentiation (Watkins et al., 2011, Zhang et al., 2011, Bivi et al., 2012). Thus, deletion of Cx43 from osteoblast precursors, mature osteoblasts, or osteocytes in mice results in increased RANKL/OPG ratio and high osteoclast numbers *in vivo*. Cx43 is also expressed in osteoclastic cells from normal mice (Ilvesaro et al., 2000) in osteoclasts from patients with Paget's disease of the bone and giant cell tumors of the bone, although the role of the connexin in these conditions has not been established (Schilling et al., 2008). On the other hand, *in vitro* studies showed that Cx43 blockade inhibits osteoclast fusion and function (Ilvesaro et al., 2000, Schilling et al., 2008, Ilvesaro et al., 2001), although whether Cx43 is also required for osteoclast differentiation and function *in vivo* remains to be determined. On the other hand, Cx37, another connexin member of the same family of gap junction proteins as Cx43, which is expressed in osteoclasts, is involved in osteoclast differentiation in mice *in vivo* (Pacheco-Costa et al., 2014). Thus, mice with global deletion of Cx37 exhibit increased

bone mass due to reduced osteoclast differentiation. The impaired osteoclastogenesis in these mice is associated with accumulation of osteoclast precursors and activation of the Notch signaling pathway.

6. CONCLUDING REMARKS

In this chapter, we described several of the signaling pathways triggered in osteoblasts, osteocytes, and osteoclasts by selected regulatory molecules, as well as by direct cell-to-cell contact. Through these interrelated signaling pathways, the generation, viability and function of osteoblasts, osteocytes and osteoclasts is controlled. Appropriate differentiation and activity of each of the bone cells is required for the maintenance of bone mass, architecture, and strength. Therefore, understanding the interrelated signaling stimuli that regulate these cells, and the intracellular signaling pathways activated, will allow for better design of strategies to improve bone mass in conditions associated with low bone mass and increased bone fragility.

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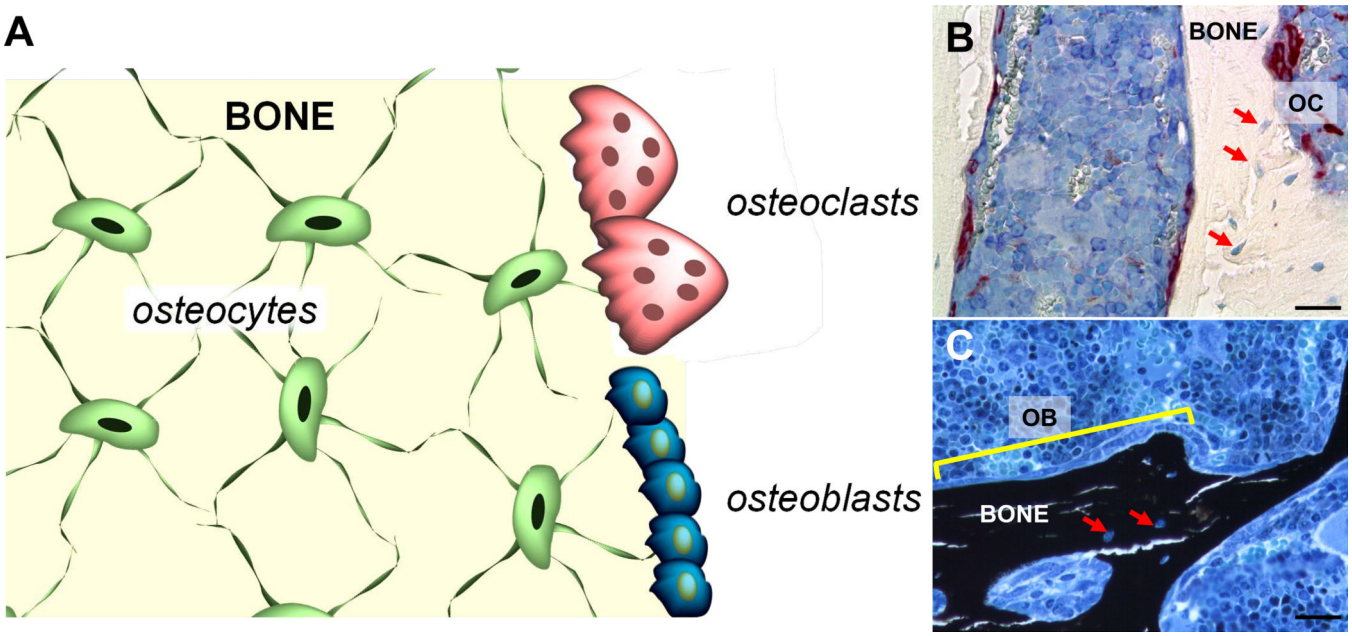

Figure 1.

A. Schematic representation of bone containing osteocytes embedded in the matrix, and osteoblasts and osteoclasts on the surface. **B** and **C.** Histologic sections of distal femur stained for **B.** TRAP (osteoclasts, OC, red) and counterstained with Toloduine blue and **C.** von Kossa (mineralized bone, black) and counterstained with McNeal. A row of osteoblasts (OB) is indicated by the yellow line. Red arrows point at osteocytes within the bone matrix. Bone sections were prepared and stained at the ICMH Histology and Histomorphometry Core, Indianapolis, IN, USA. Scale bars indicate 25μm.

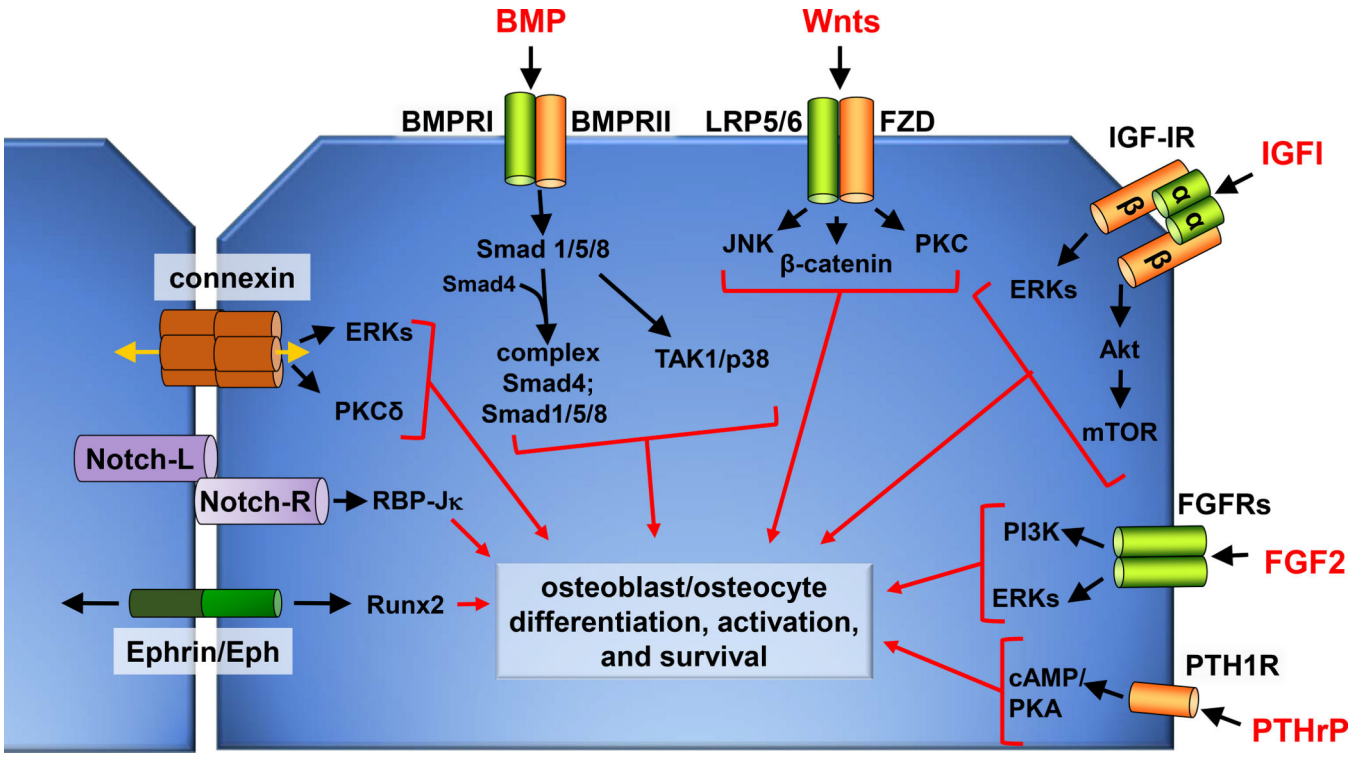

Figure 2.

Schematic representation of two neighboring osteoblastic cells, showing extracellular ligands and their corresponding receptors, and cell-to-cell signaling molecules. Selected downstream signaling molecules and the biological outcomes are also included. Notch-L: Notch ligand; Notch-R: Notch receptor.

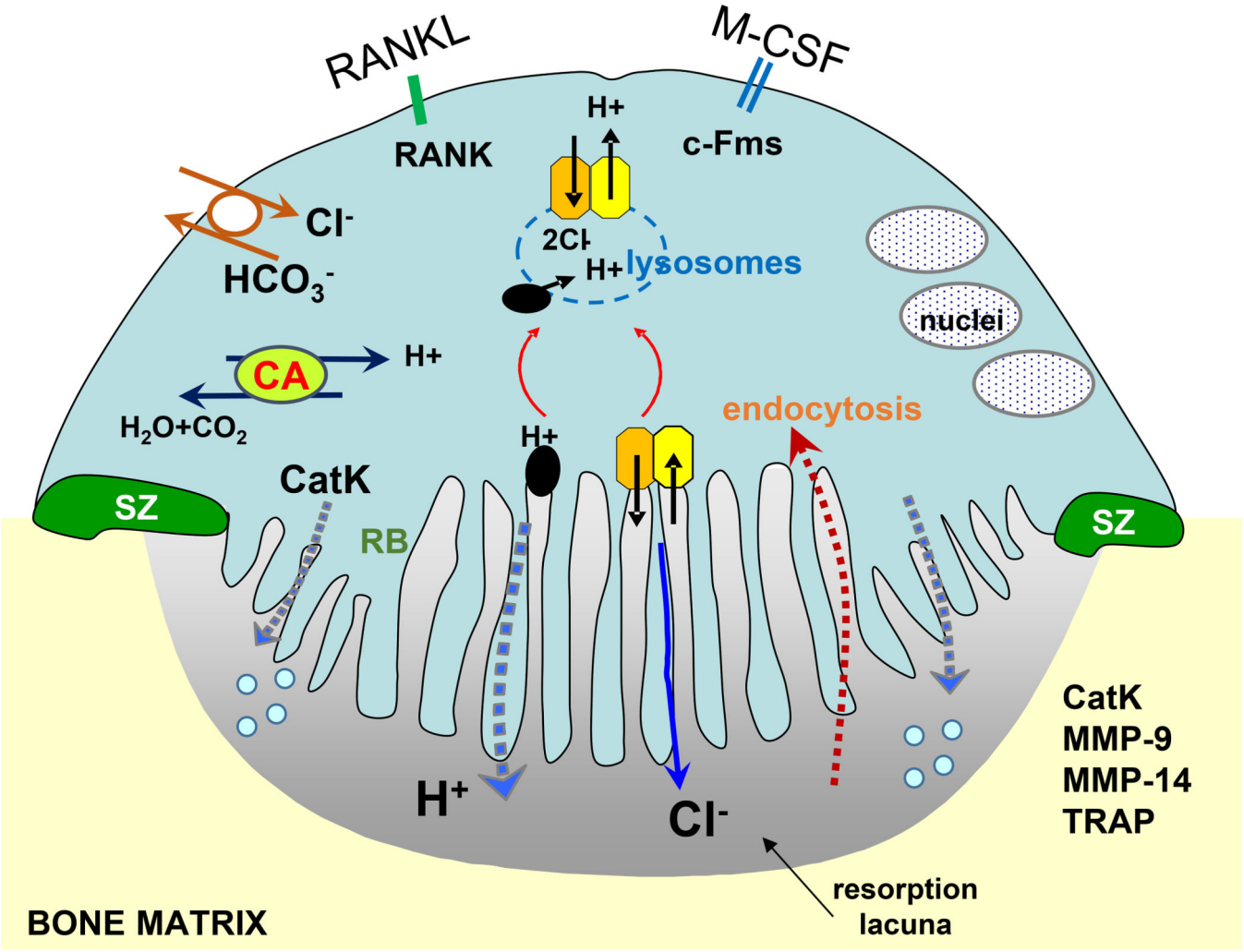

Figure 3.

Schematic representation of a resorbing osteoclast showing the sealing (SZ), ruffled border (RB) membrane, nuclei and resorption lacuna. Mature osteoclasts are polarized with the apical domain directed towards the bone surface. Protons generated by carbonic anhydrase (CA) are transported via the proton pump (black elipse) to the ruffled border membrane. A coupled basolateral bicarbonate/chloride exchanger (solid white circle) maintains electroneutrality, avoiding changes in pH and/or membrane polarization of the cell. Secretion of protons via the proton pump (black circles) and chloride ions via ClC-7 (orange) in complex with Ostm1 (yellow) acidifies the resorption lacuna. Bone dissolution occurs through the actions of secreted cathepsin K (CatK), matrix metalloproteases and TRAP. Bone degradation products are released into the bone microenvironment during cell migration, or are internalized via recycling vesicles into the cell and are secreted, or degraded via the lysosomes (blue dashed ring). RANKL binding to RANK receptor and M-CSF binding to the c-Fms receptor are also indicated.