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Pediatric Bone Health Update

Bone equilibria and disruptions

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ABSTRACT

Osteoporosis is considered a disease of adulthood, but there is increasing recognition that poor bone density during childhood can have effects decades later. To understand the pathogenesis of osteoporosis, it is important to understand normal bone maintenance and remodeling, since disruptions of these processes lead to pathologic bone. Bone maintenance is a complex and highly regulated system, consisting of several homeostatic equilibria. This article highlights three homeostatic systems. The first, the interplay between the differentiation of osteoblasts from mesenchymal stem cells and osteoclasts from hematopoietic stem cells, is the most important. Estrogen has a direct effect on the system, and its absence is pivotal. The second is a lesser-known homeostasis that functions between bone and bone marrow adipose tissue, which can insidiously drive osteoporosis. Bone marrow adipose tissue acts as a regulator of bone metabolism, negatively affecting bone formation. The third homeostatic system covered is the microbiota-gut-bone axis, where the make-up of the gut microbiome can influence a balance between osteoblastic and osteoclastic T-cells. Understanding these systems has provided avenues of study for existing and future treatments.

Key Concepts:

(1) The balance between bone formation and bone resorption is driven by factors that initiate the differentiation of mesenchymal stem cells to osteoblasts and hematopoietic stem cells to osteoclasts.
(2) Bone marrow adipose tissue is formed by adipocytes that are the result of diversion of mesenchymal stem cells from the osteoblastic differentiation pathway.
(3) The health of the gut microbiome has direct effects on the bone homeostatic processes.

Bone homeostasis

Bone maintenance is a balance of formation and resorption; on average 10% of the adult skeleton is remodeled annually [1]. The most powerful systems regulating homeostasis are the feedback loops that govern the production of bone forming and bone resorbing cells. Proliferation and differentiation of their respective stem cells are either limited or stimulated, thereby regulating the population of active bone cells. Osteoblasts, the bone forming cells, are derived from mesenchymal stem cells and comprise 4 to 6% of the total resident bone cells. They are active for about 3 months. Osteocytes are terminally differentiated osteoblasts and make up 90–95% of the resident bone cell population. Their lifespan can be up to 25 years. They have a role both in bone homeostasis and functional adaptation of bone and act as mechanosensory cells within the bone. They can also communicate with each other directly by gap junctions. Osteoclast, the bone resorbing cells, are derived from hematopoietic stem cell lines and make up the remaining 1–4% of bone cells [1–3]. Together, these cells congregate in unique temporary anatomical structures called basic multicellular units (BMU) [1,4]. The BMUs are 1–2 mm long with osteoclasts at the leading edge, osteoblasts trailing, and a central vascular capillary in between.

The undifferentiated stem cells that give rise to the osteoblasts and osteocytes reside in stem cell niches, which Ohlstein defined in 2004 as "a specific location in a tissue where stem cells reside for an indefinite period of time and produce progeny while self-renewing" [5]. The niches allow for paracrine cell signaling (communication with adjacent cells) and autocrine signaling (release of a factor by a cell that then bind cell-surface receptors of that same cell). The stem cell niches most relevant to our topic are located deep to the periosteum and in the bone marrow.

Osteoblasts

Osteoblasts are derived from mesenchymal stem cells (MSC), undifferentiated cells that also have the potential to differentiate into

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chondrocytes, myocytes, adipocytes, and fibroblasts. Factors that stimulate the MSCs to differentiate along the osteoblastic lineage are:

1) Wnt signaling – homologous wingless (wg)/integrated-1 signaling pathway, which acts as both paracrine and autocrine communication [6]. Wnt signaling can be inhibited by sclerostin (SOST), which is released by osteocytes, [7] and by dickkopf-1 (DKK-1), released by osteoblasts, creating a negative feedback loop [8]. In addition to its osteoblastogenic functions, Wnt signaling can independently suppress osteoclastogenesis and is probably the most important pathway governing osteogenesis [9].
2) BMP – bone morphogenic proteins, part of the transforming growth factor ß (TGF-ß) superfamily of multifunctional cytokines, secreted by osteoblasts and osteocytes [10]. BMP-2, BMP-6, BMP-7, and BMP-9 are the most important for bone formation [11]. The BMPs stimulate the expression of Runt-related transcript factor 2 (RUNX2) within the osteoblast progenitors [2]. RUNX2, called the master gene of osteoblast differentiation, regulates gene expression of Osterix (OSX) within the osteoblast progenitor; OSX then drives early osteoblast differentiation [12]. A mutation in the gene coding for RUNX2 underlies cleidocranial dysplasia, a condition where failure of intra-membranous ossification affects flat bones such as the clavicles [13]. BMP-3 is antagonistic to osteogenic BMPs.
3) PTH – parathyroid hormone, secreted by the parathyroid glands. Intermittent PTH secretion recruits MSCs into the osteoblastic lineage and promotes the differentiation of osteoprogenitors into mature osteoblasts [14]. PTH also downregulates the osteocytic release of sclerostin (SOST), removing an inhibitor of the Wnt signaling pathway [15]. Paradoxically, continuous exposure to PTH will lead to bone resorption [16].

Wnt signaling, BMPs, and TGF-ß all stimulate RUNX2 expression, driving mesenchymal stem cell differentiation commitment towards an osteoblastic precursor. Wnt signaling also stimulates OSX expression, which further influences pre-osteoblasts to mature into fully functional osteoblasts [17].

MSC differentiation into osteoblasts is a 3-step process (Fig. 1). A mesenchymal stem cell is stimulated by BMP and Wnt, leading to the expression and release of RUNX2 by the cell, and its differentiation into an osteoblast progenitor. Stimulation of that osteoblast progenitor by BMP, Wnt, PTH and TGF-ß leads to the expression of both RUNX2 and OSX, driving the cell to differentiate into an immature osteoblast. Further stimulation by Wnt, PTH, and TGF-ß of the immature osteoblast, and ongoing expression of RUNX2 and OSX culminates in formation of the mature osteoblast [2]. Once osteoblasts have served their function, they either undergo apoptosis, become embedded in bone and become osteocytes, or attach to the surface of bone as quiescent flat shaped bone-lining cells [2].

Prolonged inflammation, as seen in such rheumatic diseases as rheumatoid arthritis, decreases osteoblastogenesis [18]. Chronic inflammation leads to an environment of hypoxemia, low pH, and the release of pro-inflammatory cytokines. The cytokines include tumor necrosis factor- α (TNF-α), and the interleukins IL1 and IL6, all of which inhibit osteoblast differentiation. The Wnt-antagonist DKK-1 is also released from osteoblasts. One potential treatment under study is antibodies to block DKK-1, which have been demonstrated to reduce local osteoclast numbers and bone resorption in mice [19]. Another possibility is in vivo inhibition of TNF-α [20].

Not all inflammation is detrimental to osteoblastogenesis. Fractures stimulate inflammatory pathways within the fracture hematoma that initiate differentiation of MSC to osteoblasts [21]. Fractures that occur without periosteal damage heal by intramembranous ossification, directly forming bone. Fractures with periosteal damage heal by endochondral ossification, requiring a cartilaginous scaffold. Many of the same factors required for osteoblastogenesis also stimulate chondroblastogenesis [22]. A recent discovery is that chondroblasts can undergo transdifferentiation to become osteoblasts, both in physes and in fracture healing [23–25]. This presents possibilities for treating arthritis as well as producing cartilage biomaterials.

Osteoclasts

Osteoclasts are derived from hematopoietic stem cells (HSC) that undergo a complex differentiation, following either macrophage or monocyte pathways [26,27]. Factors that stimulate the HSCs to differentiate along the osteoclastic lineage are:

1) M-CSF – macrophage colony-stimulating factor, secreted by osteoblasts [28]. M-CSF influences monocytes to differentiate into osteoclastic precursors.
2) RANKL – receptor activators of nuclear factor-κB ligand. Osteocytes upregulate the migration of osteoclasts by inducing osteoblasts to express RANKL [29]. RANKL's surface receptor on the osteoclastic precursors is RANK, and their ligand-receptor binding initiates intracellular cascades that lead to the formation of such transcription factors as nuclear factor-κB (NF-κB) and nuclear factor-activated T-cells cytoplasmic 1 (NFATc1), both imperative for osteoclastogenesis [27,30].
3) NF-κB is the end product of pathways initiated by either RANKL or TNF-α binding their cell surface receptors on the osteoclastic progenitor. NF-κB enters the osteoclast nucleus to activate transcription factors, most importantly NFATc1 [31]. NFATc1 is the master switch for regulating terminal differentiation of osteoclasts, inducing within the precursors a number of genes involved in final cell differentiation [32]. Inhibition of NF-κB suppresses bone resorption by inhibiting osteoclastogenesis and promoting bone formation [33].
4) PPARγ – peroxisome proliferator activated receptor gamma. Expressed by monocytes and macrophages, PPARγ is a transcription

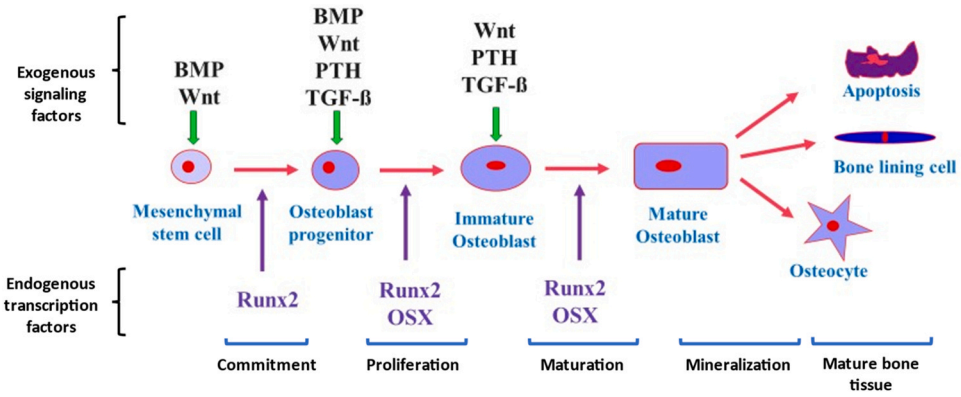

Figure 1. Stages of the osteoblast differentiation pathway. The uncommitted mesenchymal stem cell is stimulated by exogenous BMPs and the Wnt signaling pathway to transcribe RUNX2 and OSX, directing terminal differentiation in the osteoblastic direction. RUNX2 functions upstream of OSX, both of which are required for osteoblastogenesis. Red arrows are osteoblastic and osteoblastogenic, green arrows indicate stimulation by exogenous factors, purple arrows indicate stimulation by endogenous/autocrine factors. BMP, bone morphogenic proteins; OSX, Osterix; RUNX2, runt-related transcript factor 2.

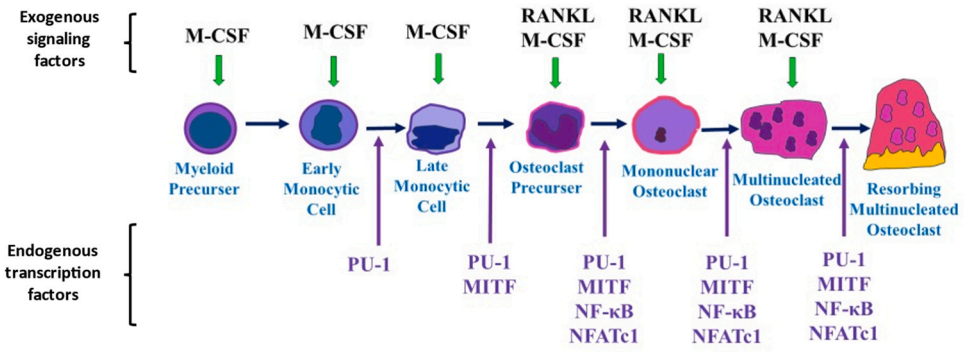

Figure 2. Stages of the osteoclast differentiation pathway. Osteoblasts release the factors M-CSF and RANKL which stimulate hematopoietic stem (myeloid precursor) cells to transcribe several factors, most importantly PU-1, MITF, NF-κB, and NFATc1. These factors are all required for development of a mature osteoclast. Black arrows are osteoclastic and osteoclastogenic, green arrows indicate stimulation by exogenous factors, purple arrows indicate stimulation by endogenous/autocrine factors. M-CSF, macrophage colony-stimulating factor; MITF, microphthalmia-associated transcription factors; NFATc1, nuclear factor-activated T-cells cytoplasmic 1; RANKL, receptor activators of nuclear factor-κB ligand.

factor that both promotes differentiation of HSCs to mature osteoclasts and inhibits osteoblastogenesis, stimulating MSCs to differentiate into adipocytes instead [34,35].

5) PU.1 is a transcription factor, likely responsible for the earliest established event in osteoclastogenesis; its genetic absence can lead to osteopetrosis, a sclerotic bone disease caused by arrested bone resorption [27,36].
6) MITF – microphthalmia-associated transcription factors are essential for driving the differentiation from mononuclear osteoclast precursors to multinucleated osteoclasts [37].

In the multistep transformation from MSC to osteoclast, the initial 3 transitions are from myeloid precursor to early monocytic cell, to late monocytic cell, then to osteoclast precursor (Fig. 2). Up to this point in the differentiation, few factors are necessary. M-CSF is present for each transition, endogenous PU.1 becomes essential starting at the transition from early to late monocyte, and endogenous MITF is an additional transcription factor leading up to the osteoclast precursor. All 3 factors are also needed for each of the subsequent differentiation steps that follow [27].

The next transition, with additional stimulation from exogenous RANKL and endogenous NFκB and NFATc1, sees the osteoclast precursor become a mononuclear osteoclast. These same transcription factors are instrumental in further maturation of the mononuclear osteoclast to a multinuclear osteoclast, and again at the final step leading to a fully formed resorbing multinucleated osteoclast [27].

The RANKL/RANK/OPG system is an important regulator of osteoclastogenesis [38](Fig. 3). Osteoprotegerin (OPG), a factor secreted by osteoblasts, competitively binds RANKL in the extracellular space. The RANKL-OPG complex prevents RANKL from binding to its cell surface receptor RANK, thwarting stimulation of osteoclast precursors and mature osteoclasts. As such, OPG acts as a decoy receptor to RANKL to prevent RANK stimulation. The balance between RANKL and OPG is a prime factor governing osteoclastogenesis, and an important communications structure between osteoblasts and osteoclasts [39]. Estradiol also influences the RANKL/OPG balance, inhibiting osteoclastic differentiation [40].

Osteocytes

At the end of their life cycle, osteoblasts either undergo apoptosis or become embedded within the developing bone and differentiate into osteocytes [2]. A small subset of osteoblasts follows a third pathway to become bone-lining cells, which facilitate osteoclastic attachment to bone [1,41]. Osteocytes are the most abundant cells in bone tissue. They act as mechanoreceptors, sensing the prevailing stress on bone and tuning the remodeling process accordingly. They have a stellate morphology, similar to the nervous system's dendritic network. Osteocytes thereby communicate with fellow osteocytes, as well as osteoblasts and osteoclasts, by direct cellular contact via gap junctional signaling, as well as by paracrine signaling [1,42].

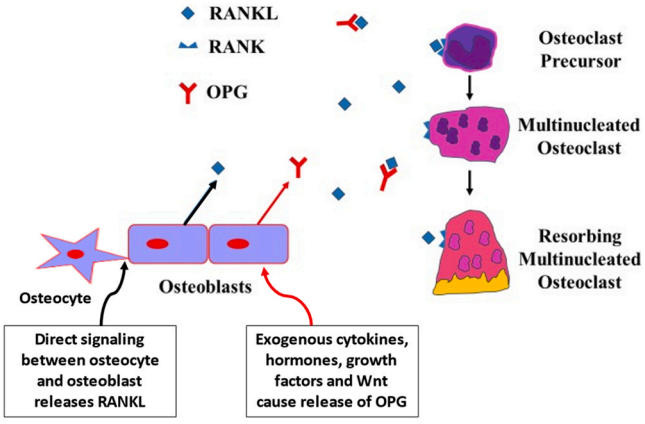

Figure 3. The RANKL/RANK/OPG regulating system. Osteoclast differentiation requires RANKL to bind the cell surface receptor RANK. RANK is present on the cell surface of osteoclast progenitors as well as mature osteoclasts. When RANK binds RANKL, an intracellular cascade leads to the transcription of factors such as NF-κB, NFATc1, and MITF. Osteocytes directly induce osteoblasts to express RANKL. Osteoblasts also release osteoprotegerin (OPG) in response to cytokines, hormones, growth factors and Wnt signaling. OPG then competitively binds RANKL, preventing formation of the RANKL/RANK complex. Red arrows and factors are osteoblastic and osteoblastogenic, black arrows and blue factors are osteoclastic or osteoclastogenic. MITF, microphthalmia-associated transcription factors; NFATc1, nuclear factor-activated T-cells cytoplasmic 1; OPG, Osteoprotegerin; RANKL, receptor activators of nuclear factor-κB ligand.

Osteocytes regulate the activities of the osteoblasts and osteoclasts. They can stimulate osteoblastogenesis by releasing prostaglandin E2, growth factors, and glycoproteins [42]. In contrast, osteocytes can also down-regulate osteoblastogenesis by releasing sclerostin (SOST) to inhibit the Wnt signaling pathway, [43] or inhibit osteoblast maturation by inducing the notch signaling pathway [44]. The notch signaling pathway, considered the third pathway that is important for MSC differentiation into osteoblasts, behind BMP and Wnt, is a microcosm of the complexity of bone regulation. Depending on the circumstances, notch signaling can act to enhance or inhibit osteoblastogenesis [17,45]. Lastly, osteocytes can induce osteoclastogenesis by secreting RANKL or inducing osteoblasts to release RANKL (Fig. 4) [29,46,47].

Estrogen and bone formation

Osteoporosis related to estrogen deficiency is one of the best-known examples of acquired bone pathology. Estrogen plays an important role in bone homeostasis, particularly in inhibiting osteoclastic functions (Fig. 5)[48]. Estrogen can block osteoclastogenic signaling pathways by binding its receptor, estrogen receptor-α (ERα), on the osteoclasts' surface or within the cytoplasm. Within the cell the complex then binds Traf6, a component of the RANKL signaling pathway, interrupting

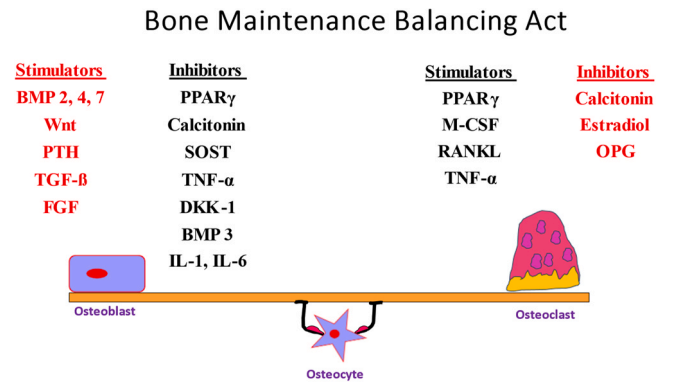

Figure 4. Osteocytes' central role in bone maintenance. Many important functions of bone maintenance homeostasis are regulated by osteocytes, primarily by controlling the rate of osteoblastogenesis and osteoclastogenesis. On the left are listed the stimulating and inhibiting factors exogenous to osteoblasts. On the right are the exogenous factors stimulatory or inhibitory to the differentiation of osteoclasts. Some of the factors are hormonal (PTH, estrogen), others have more of a paracrine function. The osteocyte is responsible for the release, either directly or indirectly, of many of the listed factors, but not all of them. Factors in red are osteoblastogenic, those in black are osteoclastogenic. FGF, fibroblast growth factor; PTH, parathyroid hormone.

formation of NF-κB and NFATc1 [27,49]. Similarly, estrogen modulates the activity of NF-κB, inhibiting it from binding promoters within the developing osteoclast's cell nucleus [1,50]. Osteoclast differentiation and osteoclastic bone resorption are further inhibited by estrogen's induced expression of the calcium transport proteins transient receptor potential vanilloid 5 and 6 (TRPV5 and TRPV6) [51,52]. Calcium is released as part of the bone resorption process and TRPV5 and 6 are important cell surface ion channels transporting calcium within the osteoclast. RANKL induces oscillatory calcium concentrations within the osteoclast, activating the NFATc1 pathway. Paradoxically, underexpression (genetic mutation) or over-expression of the calcium transport proteins can inhibit osteoclastogenesis and proper osteoclast functioning. Estrogen increases expression of TRPV5 and TRPV6 which then leads to apoptosis of the osteoclast [53,54].

Estrogen also has a direct effect on osteoblastogenesis by both activating the Wnt signaling pathway and upregulating BMP signaling [55]. By binding its receptor ERα in the cytoplasm, the estrogen-ERα complex can potentiate the Wnt and BMP signaling cascades in the extranuclear space [56]. It can also stimulate Wnt mediated transcription within the nucleus. These actions promote mesenchymal stem cells to differentiate from pre-osteoblasts into mature osteoblasts, diverting them from the adipocytogenesis pathway. By binding its receptor, estrogen can mediate the RANKL/OPG balance by both suppressing the production and action of RANKL and promoting OPG expression, shifting emphasis to osteoblastogenesis over osteoclastogenesis [57].

Clearly, the loss of estrogen leads to an imbalance of osteoclast/osteoblast formation. Understanding estrogen's role in bone homeostasis and the effects of its loss has led to potential treatments. Estrogen replacement therapy has been shown to be beneficial for postmenopausal women; the addition of progesterone reduces the risk of endometrial hyperplasia or carcinoma of the uterus [58]. Selective estrogen receptor modulators (SERM) are a class of drugs that act as estrogen agonists on the estrogen receptors; several are already in use [48]. Denosumab, a monoclonal antibody to RANKL, acts as its inhibitor and has been found safe and effective in reducing the risk of fractures [59].

Bone marrow adipose tissue

A common experience of operating on children with chronic neuromuscular disorders is finding that their bone quality is poor. When drilling or osteotomizing the bone, it is often remarkably oily [60]. This is the result of the active process of bone marrow adipose tissue (BMAT) accumulation. The dysregulated equilibrium between osteoblastogenesis and adipocytogenesis within the bone marrow is the second most important imbalance leading to osteoporosis [61]. Age-related osteoporosis and post-menopausal osteoporosis favor adipocytosis, as do the inflammatory environments often seen in osteoporotic conditions [62]. Paradoxically, both excess calories (obesity) and caloric restriction (anorexia) can induce BMAT [63].

Besides the osteoblastic pathway, mesenchymal stem cells (MSCs) can also differentiate into other cells lines, including adipocytes. They are stimulated to do so by some of the same factors that also stimulate osteoclastogenesis, including PPARγ and the CAAT enhancer binding

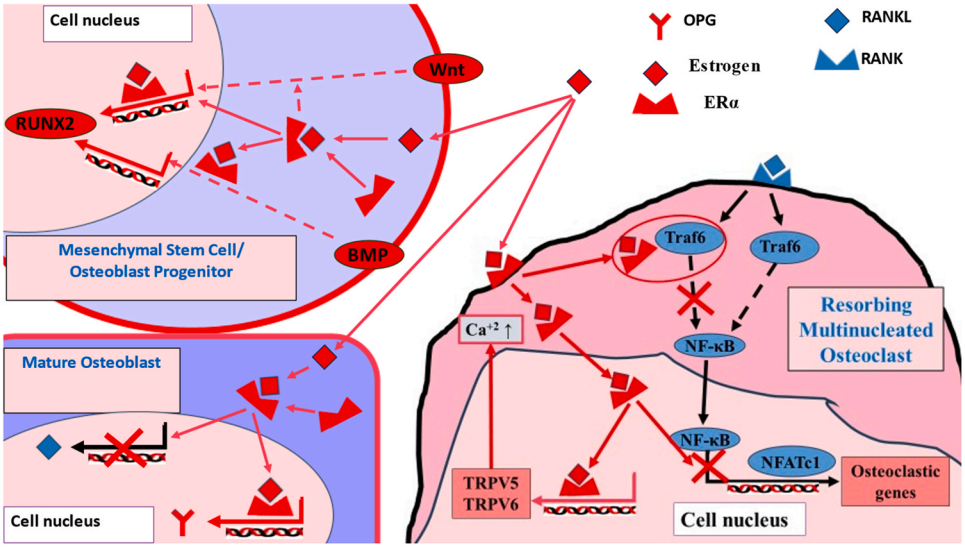

Figure 5. Estrogen's role in bone homeostasis. The RANKL-RANK signaling pathway within the osteoclast requires the factor Traf6 as an intermediary. On the osteoclast's surface or in its cytoplasm, estrogen binds its receptor, ERα and the complex interrupts the pathway by sequestering Traf6, preventing formation of NF-κB and NFATc1, and thereby inhibiting both osteoclastogenesis and osteoclast functioning. The estrogen-ERα complex also transits into the cell nucleus to inhibit NF-κB from binding promoters within the osteoclast's nucleus, and separately causes overexpression of the calcium-transport proteins TRPV5 and TRPV6 within the osteoclast. The latter leads to apoptosis of the osteoclast. Estrogen stimulates osteoblastogenesis by enhancing both Wnt and BMP signaling cascades in the mesenchymal stem cells and the osteoblast progenitor cells. Estrogen-ERα can potentiate the Wnt and BMP signaling pathways in the extranuclear space directly and also function as an adjunct to transcription. In the osteoblasts, estrogen suppresses RANKL formation while promoting OPG expression. Note that the estrogen can directly traverse the cell membranes to bind its receptor in the cytoplasm, or can act via specific cell surface receptors. Red factors and arrows are osteoblastic and osteoblastogenic, blue factors and black arrows are osteoclastic or osteoclastogenic. Dashed arrows indicate multistep signaling pathways. Right angled arrows indicate transcription, with relevant transcription factors positioned above. BMP, bone morphogenic proteins; NFATc1, nuclear factor-activated T-cells cytoplasmic 1; OPG, Osteoprotegerin; RANKL, receptor activators of nuclear factor-κB ligand.

proteins (C/EBP) [64]. In essence, PPARγ and C/EBP serve as the master transcription factors for committing MSC to the adipocyte differentiation pathway, in the same way that RUNT and OSX function to drive MSCs to commit to osteoblastogenesis. Other stimulating factors include a number of micro RNAs (miRNAs) [61]. miRNAs are short RNA chains of 19–25 nucleotides in length, of which some are osteogenic while others are adipogenic. The adipogenic miRNAs can suppress expression of RUNX2, thereby suppressing osteoblastogenesis; they are highly expressed with aging.

The BMAT adipocytes are well adapted towards self-preservation, pursuing suppression of osteoblastogenesis and proliferation of adipocytes. To suppress osteoblastogenesis, adipocytes release BMP receptor antagonists, blocking the effects of BMP [65]. The release of PPARγ and C/EBP by myeloid cells drives mesenchymal stem cells to commit to adipocytic differentiation rather than osteoblastic [64]. They also stimulate the adipocytes to release TNF-α and pro-inflammatory cytokines (interleukins IL-1ß and IL-6) that then stimulates MSCs to differentiate along the adipocytosis pathway, and in parallel enhance the differentiation of HSCs into osteoclasts [61]. The result is an inverse relationship between BMAT and bone mineral density (BMD), contributing towards a downward spirals of bone health. To worsen the situation, PPARγ, C/EBP, and the TNF-α released by the BMAT adipocytes, lead to an upregulated expression of RANKL by osteocytes and osteoblasts, favoring osteoclastogenesis [66]. Moreover, preadipocytes can also express RANKL when exposed to C/EBP (Fig. 6)[67]. Interestingly, mice with genetically deficient RANKL in bone marrow adipocytes have drastically increased trabecular bone [68].

There are some promising options on how to reverse the aggregation of BMAT and thereby improve bone health. Patient-based treatments include exercise and mechanical loading of bones [69]. Medications that show promise include metformin, an antidiabetic type II medication that reverses high fat-diet induced osteoporosis in mice, [70] and romosozumab, a monoclonal antibody to sclerostin (SOST), that blocks SOST from inhibiting Wnt pathway [71]; the latter has been approved for post-menopausal osteoporosis. Under experimental consideration is bisphenol-A diglycidyl ether (BADGE), a PPARγ antagonist that has been shown to increase bone mass in mice [72]. Other experimental protocols have to do with micro RNAs, local administration of either pro-osteogenic miRNAs or of antibodies/binding factors to pro-adipogenic miRNAs; both strategies have enhanced osteogenic differentiation of bone marrow stem cells in mice [73,74].

Gut microbiome, pro-, pre-, and post-biotics, and bone health

The 1000 different resident bacterial species in the gut microbiome are referred to as the gut microbiota, and contains 150 times more genes than the human genome. These species have co-evolved with the human genome, and are often called human's second gene pool. There is an ongoing interaction between the body's systems and the gut microbiota, such as gut endocrine cells that are affected by, and responsive to, the gut microbiota. Not surprisingly, the gut microbiome has an effect on bone health as well, and can drive bone remodeling both in the direction of bone absorption or bone formation [75,76].

The role of T-cells in gut flora is closely tied to bone health.

1) $CD4^+$ T-cells and $CD8^+$ T-cells are "naïve" T-cells that can differentiate into any number of different subsets once exposed to an antigen. Each subset has a distinct aim of orchestrating and mobilizing other cell types to effectively clear invading pathogens.
2) Some $CD4^+$ T-cells can secrete TNF-α in the colon as well as secrete TNF-α and IL-1 in bone marrow, making these $CD4^+$ T-cells osteoclastogenic [75].
3) Th17 cells - T helper 17 cells. These are activated $CD4^+$ T-cells which were exposed simultaneously to TGF-ß and IL-6 and underwent differentiation into Th17 cells. The Th17 cells produce a powerful inflammatory interleukin, IL17, as well as RANKL and TNF-α that, among other effects, inhibits osteoblastosis and spurs on osteoclastogenesis [77].
4) $CD8^+$ T-cells can secrete OPG and interferon-gamma (IFN-γ), which both inhibit bone loss [78].
5) Treg cells - regulatory T cells. These are derived from both $CD4^+$ and $CD8^+$ T-cell lines. They induce $CD8^+$ T-cells to activate the Wnt signaling pathway, which both suppresses osteoclastogenesis and stimulates osteoblastogenesis [79]. Treg cells also reduce colonic inflammation [80].

The relative balance of the Th17 cells osteoclastogenic properties and Treg cells osteoblastogenic properties is a third important homeostatic balance for bone maintenance [81]. Germ-free mice provide an illustrative example. They are mice born with, and maintained with, a sterile gut, and are found to have lower growth rates and weight compare to wild type mice, but also have increased bone mass and decreased numbers of osteoclasts in trabecular bone [82]. They are found to have decreased $CD4^+$ T-cell populations, which leads to

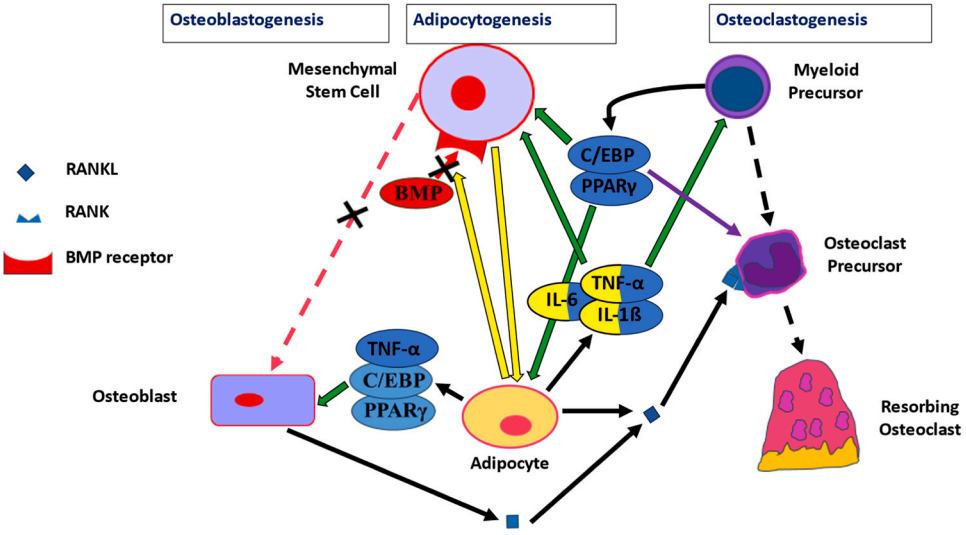

Figure 6. Bone marrow adipocyte-osteoclast co-stimulation. Hematopoietic stem cells release PPARγ and C/EBPs, activating transcription of osteoclastogenic factors such as NFATc1, and also driving commitment of the mesenchymal stem cell towards the adipocytic pathway. The adipocytes release RANKL and inflammatory cytokines such as TNF-α, IL-1β, IL-6, which significantly promote osteoclast differentiation and function. TNF-α, IL-1β, and IL-6 also stimulate MSCs to differentiate along adipocytic lines. The adipocytes release a BMP receptor antagonist, inhibiting BMP's influence on the mesenchymal stem cell to differentiate along osteoblastic lines. PPARγ, C/EBP, and TNF-α stimulate osteoblasts to also release RANKL, with further osteoclastic effects. Red factors and arrows are osteoblastic and osteoblastogenic, blue factors and black arrows are osteoclastic or osteoclastogenic, yellow factors and arrows are adipocytogenic, green arrows indicate stimulation by exogenous factors, purple arrows indicate stimulation by endogenous/autocrine factors. Dashed arrows indicate a multi-step process. BMP, bone morphogenic proteins; MSCs, mesenchymal stem cells; NFATc1, nuclear factor-activated T-cells cytoplasmic 1; PPARγ, peroxisome proliferator activated receptor gamma; RANKL, receptor activators of nuclear factor-κB ligand.

decreased TNF-α in the colon and decreased TNF-α, IL-1, IL-6 in bone. If the germ-free mice are ovariectomized, their germ-free status is protective against trabecular bone loss. In contrast, introduction of gut microbiota to the germ-free mice restores normal bone marrow immune status but decreases bone mass to "normal" [82,83].

Studies over nearly two decades have shown that microbiota imbalance (dysbiosis) can underlie metabolic diseases through metabolic endotoxemia [84]. Subsequently, the focus has broadened to include studies seeking to determine mechanisms to correct imbalances in the gut microbial community. Pro, pre and postbiotics have all been found effective in correcting dysbiosis, and provide current and potential future options for osteoporosis treatment.

1) Probiotics were defined by Salminen as "a live microbial food ingredient that is beneficial to health" [85]. For humans these are primarily the *Lactobacillus* and *Bifidobacillus* species.
2) Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve host health" [86]. These can include fiber-rich diets (artichoke, garlic, leek, dandelion greens, banana, onion, chicory), or augmenting the diet with inulin and lactulose supplementation products.
3) Postbiotics are useful metabolites produced by gut flora, mostly as a result of the gut microbiota digesting the prebiotic materials, but postbiotics can also be ingested as nutritional supplements [79]. These metabolites include hydrogen sulfide, vitamins B and K, some amino acids, and short chain fatty acids (SCFA). The SCFAs are produced in the gut microbiome by fermentation of dietary (vegetable) fibers, or catabolism of amino acids and lactate [79]. The main beneficial SCFAs produced are butyrate, propionate and acetate [87].

The aspect of the gut microbiome-intestinal immune system relationship that most affects bone is largely based on butyrate and propionate, and to a lesser extent acetate, which facilitate the generation of the anti-inflammatory Treg cells. The best studied is butyrate, which directly promotes $CD4^+$ T-cells to differentiate into Treg cells [80,88]. Treg cells are induced in the intestines, spleen, and bone marrow, and they in turn stimulate the Wnt signaling pathway, driving osteoblastogenesis (Fig. 7)[89]. Butyrate and propionate also inhibit osteoclast differentiation; supplementation of these SCFAs to ovariectomized mice reduced the expected osteoclast formation and bone loss [90]. To summarize, beneficial gut bacteria (probiotics) digest otherwise indigestible food stuffs (prebiotics), creating metabolites (postbiotics) that enhance Treg cell formation. By increasing the proportion of Treg cells relative to Th17 cells, the balance is tipped in the direction of bone preservation [76].

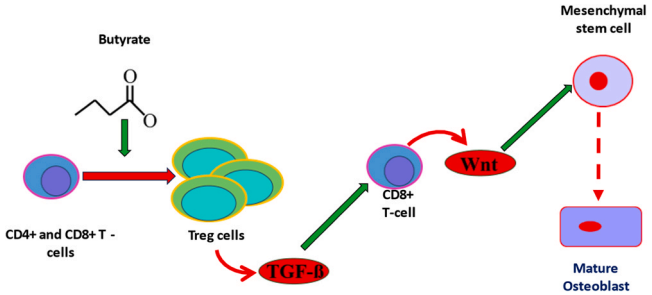

Figure 7. The probiotic bacteria Lactobacillus rhamnosus, as a dietary supplement, augments butyrate levels in intestinal tissue and serum. Butyrate augments the differentiation of naïve helper CD4+ cells into Treg cells in the intestine, spleen, and bone marrow. Tregs cells activate a signaling pathway that increases the production of TGF-β by Treg cells, stimulating CD8+ cells to transcribe the osteogenic Wnt ligand. Wnt signaling is activated in bone marrow stromal cells, causing their proliferation and differentiation of those stomal cells into osteoblasts. The expansion of the osteoblastic population results in increased bone formation and improved bone structure. Red factors and arrows are osteoblastic and osteoblastogenic, green arrows indicate stimulation by exogenous factors, dashed arrows indicate a multi-step process. TGF-β, transforming growth factor β.

Other studies on mice have illuminated much about the gut microbiome and the benefits of pro, pre and postbiotics. In one study, ovariectomized mice experienced an increase in gut permeability and overall inflammation [91]. That led to an increase in TNF-α, RANKL, and IL-17 release in bone and in the gut, which then drove osteoclastic differentiation. Probiotics, particularly *Lactobacillus rhamnosus* or a *Lactobacillus/Bifidobacterium* mix, decreased gut permeability, thereby decreasing bowel and bone marrow inflammation, and completely protecting against bone loss in the ovariectomized mice [91]. Other authors reported on the phenomena of osteonecrosis of the femoral heads of mice following exposure to the glucocorticoid methylprednisolone. Methylprednisolone was found to cause dysbiosis in the mice microbiome, resulting a loss of a *Lactobacillus* species that ordinarily provided extracellular vesicles containing proangiogenic, pro-osteogenic and antiapoptotic proteins, all of which protect the femoral heads. Providing the mice with oral probiotics reversed the methylprednisolone induced dysbiosis and protected the femoral heads [92]. In another study, mice were forced into obesity via a high fat diet, and experienced bone resorption and bone loss. An increase in colonic inflammation was associated with the diet. Treating the obese mice with a probiotic or the postbiotic indole-3-propioinc acid (IPA, a tryptophan metabolite) prevented gut inflammation and improved mineralization of bone. The probiotic, a *Lactobacillus/Bifidobacillus* mix, led to increased IPA levels in the gut and plasma. The IPA promoted osteoblast differentiation by enhancing mitochondrial metabolism within osteoblasts [93]. Lastly, a study focused on insulin-like growth factor 1 (IGF-1), a hormone that manages the effects of growth hormone (GH). IGF-1 and GH together promote normal bone growth. Antibiotic treatment of wild-type mice caused a decrease in IGF-1 and subsequently of bone mass. Supplementing SCFAs to the diet of the antibiotically treated mice restored the IGF-1 levels, and the bone mass [82].

Currently, there are only a few studies that address the gut microbiota-gut-bone axis and bone health in humans. In a meta-analysis of the effectiveness of probiotic supplements for treatment of postmenopausal osteoporosis, 5 human randomized controlled trials (RCT) were identified, for a total of 497 women, followed for 6–12 months. Probiotics were associated with preserved bone mineral density in the lumbar spine, but not in the hips. Levels of type 1 collagen breakdown products, either cross-linked C-telopeptide in the serum or cross-linked N-telopeptide in the urine, were lower in the probiotic treated cohorts compared to the control groups, indicating less bone turnover for the women undergoing treatment [94]. The authors concluded that more studies were needed to validate the results, but recommended probiotics supplements to postmenopausal women to improve bone status. The only available study on children was a RCT of 100 adolescents, 50% female, randomized to a placebo group or a group receiving supplementation of an inulin product. The study group had better calcium absorption at 8 weeks (8.5%) and at one year (6%); at 1 year they also had a greater increase in whole body bone-density and bone-mineral content compared to the control group [95].

Vitamin D

The life cycle of vitamin D (also referred to as "calciferol") starts with either endogenous 7-dehydrocholesterol that is converted to vitamin D3 in the skin or exogenous vitamin D2 and D3 that are absorbed in the intestine. From there, conversion to 25-hydroxyvitamin D occurs in the liver by 25-hydroxylases, respectively becoming 25(OH)D2 or 25(OH)D3. 25(OH)D has a half-life of 2–3 weeks. In the kidney, and to some extent in the peripheral tissues, 25(OH)D is hydroxylated to the

active 1, 25 dihydroxyvitamin D ($1,25(OH)_2D$), which has a half-life 4–6 hours. The vitamin D-binding protein (DBP) transports 25(OH)D and $1,25(OH)_2D$ and their metabolites throughout body.

The vitamin D receptor (VDR) can be found in multiple tissues, including bone, cartilage, intestine and kidney, and exists in 2 forms, as membrane-associated (mVDR) and nuclear (nVDR) receptors. mVDR binds $1,25(OH)_2D$ which activates a signaling pathway resulting in increased intracellular uptake of calcium via calcium channels, and a release of calcium from the endoplasmic reticulum. On the other hand, the nVDR-$1,25(OH)_2D$ complex binds the retinoid X receptor (RXR) within the cytoplasm which facilitates the complex' translocation into the nucleus, where it activates more than 1000 target genes [96]. Among its many functions, vitamin D is best known for facilitating the transport of ingested calcium across the gut mucosal border and into the blood stream. It also works in concert with parathyroid hormone to maintain plasma calcium levels by releasing calcium from bone as needed [97]. Vitamin D has a complicated relationship with osteoblastogenesis, on the one hand acting as a stimulator of osteoblast differentiation, on the other inhibiting the process [98,99]. Vitamin D can also enhance osteoclast formation from peripheral monocytes by stimulating release of RANKL from osteoblasts [100].

Bisphosphonates

Bisphosphonates are a class of chemical-analogs to inorganic pyrophosphate, and as such bind to hydroxyapatite crystals in actively remodeling bone. By doing so, they both inhibit calcification, but more importantly inhibit hydroxyapatite breakdown and bone resorption [101]. The first-generation bisphosphonates, of which etidronate is best known, are non-nitrogen based. Their by-products are cytotoxic to osteoclasts and accumulate within the cells during bone resorption, leading to osteoclast apoptosis [102]. The second and third-generation bisphosphonates are nitrogen containing (eg, alendronate, pamidronate, and zoledronate), and also inhibit bone resorption by osteoclast apoptosis. They do so differently from the first-generation chemicals, by blocking the osteoclasts from producing sterols, which inhibits post-translational modification of key proteins, ultimately leading to cell death [103].

Conclusions

Bone maintenance is a complex system of feedback loops and cell-cell communication, which when functioning well preserves bone health. Presented here are but three of the many intricate homeostatic structures, with many more within the signaling pathways and at the level of gene transcription of factors. Understanding these interactions will lead to interventions to improve bone health. A better understanding of post-fracture inflammation may lead to other factors that can artificially stimulate bone healing. Chronic inflammatory disorders might benefit from further study of antibodies to block DKK-1 or inhibitors of TNF-α. Post-menopausal osteoporosis research may provide treatments for girls with delayed puberty to help them achieve maximal bone health.

Besides the pharmacological treatments available or under study, there are also several simpler strategies that can be suggested to patients. Intake of necessary vitamins and minerals in diet or through supplementation is important. Exercise and mechanical loading can reverse bone marrow adipose tissue deposition. Vibrating plates have had success in animal models for increasing bone stock, but those results have not yet been replicated in humans [104,105]. Supplementing with probiotics (*Lactobacillus* and *Bifidobacillus* species), pre/post biotics (such as inulin and butyrate supplements), or merely a high fiber diet, are relatively accessible and facile prophylactic options. The importance of bone health for all children cannot be overstated.

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