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Skeletal morphofunctional considerations and the pituitary-thyroid axis

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1. ABSTRACT

The past decade has unraveled novel molecular mechanisms not only of skeletal remodeling, which is the process by which the skeleton is restructured throughout adult life, but also the precision by which the skeleton is put together during embryogenesis and later modeled during growth. It is now possible to delete single genes in individual cells and during specified periods of life. This has allowed us to pin down specific molecular events that underlie individual cellular processes, and also importantly, to identify molecular defects underlying disorders of skeletal morphogenesis and remodeling. Particularly novel has been the demonstration of cross-talk, some of which is humoral, between the skeleton and organs as diverse as the brain, pituitary, and even adipose tissue and pancreas. The current review describes these molecular mechanisms in relation to the way thyroid hormones, and the pituitary hormone thyrotropin (TSH), regulate skeletal morphogenesis and remodeling.

2. INTRODUCTION

Von Recklinghausen (1890) first reported an association between bone loss and hyperthyroidism. Since then, it has become accepted by virtue of rich anecdotal experience and clinical research that thyrotoxicosis is associated with a high-turnover osteoporosis. Accelerated bone resorption is not compensated by a coupled increase in bone formation, resulting in net bone loss, and an increased fracture rate. In particular, excessive resorption of the cortical bone in hyperthyroidism results in an increased risk (~1.8 fold) of hip fractures. Furthermore, recovery of bone loss after correction of the thyroid overactivity, even in younger patients, is never complete, leaving such individuals at an increased fracture risk for the remainder of their lives. In addition, therapeutic suppression of TSH for thyroid cancer is associated with increased osteoporosis, again mainly in the post-menopausal period.

We have shown recently that deficiency of TSH receptors (TSHR) is associated with a high-turnover osteoporosis in mice, even in a euthyroid haploinsufficient state. This suggests that bone loss due to hyperthyroidism may also result from low TSH levels; this sets forth a new clinical paradigm where pituitary hormones play a critical role in bone loss.

Despite the known deleterious effects of thyroid hormones on the adult skeleton, it is clear that skeletogenesis, both during development and growth, is tightly regulated by thyroid hormones. The absence of thyroid hormones, for example in congenital forms of hypothyroidism, as well as genetic mouse models of hypothyroidism, results in stunted growth and development. Evidence to date suggests that thyroid hormones regulate endochondral bone formation directly.

This review focuses on our current and evolving understanding of embryonic skeletal development and bone remodeling, a process by which new bone replaces old bone, in the adult. After detailing the two components of bone remodeling, we explore how mouse genetic studies relate these processes to the thyroid and pituitary axis. Finally, the clinical implications of the new data are discussed.

3. LIMB PATTERNING AND JOINT FORMATION

Limb patterning in the skeleton is a critical developmental processes for all mammals (1). The process of skeletal morphogenesis begins with the migration of lateral mesoderm cells into the nascent limb bud to produce a mesenchymal cell population. The determination and patterning of these cells along the proximal-distal axis creates pre-skeletal mesenchymal condensations. There is solid evidence that the *Hox* gene family, *Hox8* through *Hox13,* exerts supremacy in determining the overall skeletal pattern. A triple *Hox10* mutant with all six paralogous alleles missing lacks the femur, while a triple *Hox11* mutant lacks the tibia and fibula (2). Likewise, the conditional ablation of all *Hoxa* and *Hoxd* functions causes early patterning arrest and shortened limbs[3]. This phenotype results from the down-regulation of a *hedgehog* family member, *Sonic hedgehog (Shh) (*3), while both *Hox* and *Shh* can be negatively regulated to prevent inappropriate patterning by specific microRNAs (4).

Complex interactions between bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), epidermal growth factor (EGF), the wingless-ints (Wnts), and patterning factors including the *Pax,* forkhead-helix and homeodomain families determine the array of shapes and sizes of skeletal elements (5). Early limb specification appears to require the T-box genes *Tbx4* and *Tbx5*; mutations of which in the latter cause Holt-Oram syndrome typified by forelimb abnormalities (6). Likewise, several BMPs, such as BMP-2, -4 and -7 regulate the dorso-ventral and proximo-distal axes, respectively, by interacting with Wnt7a and FGF-8. In contrast, signaling through several distinct FGFs, namely FGF-4, FGF-9 and FGF-17 that allow continued *Shh* production by down-regulating the BMP antagonist gremlin determines limb size (7,8). Even with these details known to date, precise mechanisms that integrate this molecular diversity to specify skeletal patterns with such exquisite intricacy remain poorly understood. It has emerged only very recently that the exactness in digit patterning is achieved through precise regulation of spatial and temporal gradients of *Shh* within a limb bud (9).

Whether there is a predetermined genetic program – a "*Hox* code" – that specifies the location of joints is also not known (10). It was proposed that segmental demarcations in *Hox* gene expression predetermine the positioning of future joints. However, the interzone, the very first mesenchymal representation for future joint formation, does not correspond with such *Hox* boundaries. Thus, what determines the address of interzone cells remains a mystery. The interzone is nonetheless unique in being composed of highly condensed mesenchymal cells interconnected by gap junctions (10). It specifies future joint elements, such as the capsule, synovial lining and articular cartilage, each structure arising from ultrastructurally distinct subsets of cells.

Established molecular signals that dictate the conversion of the interzone cells into joint structures include TGFβ superfamily members BMP-2, -4, GDF-5 and GDF-6; Wnt4, Wnt14 and Wnt16; the BMP antagonists noggin and chordin; FGF2, FGF4 and FGF13; connexins; the transcription factors *Cux-1* and *Erg;* and other molecules, such as stanniocalcin (11). The Wnts and noggin represent early anti-chondrogenic signals that maintain the mesenchymal nature of the interzone, a feature that is required for joint formation (12). Interestingly, while noggin$^{-/-}$ mice expectedly do not form joints, chordin$^{-/-}$ mice have normal joints, for unclear reasons. Likewise, mice lacking both GDF-5 and GDF-6 and humans with GDF-5 mutations display multiple joint defects (13). Another provocative hypothesis, based on evidence that mice lacking *Indian hedgehog (Ihh)* also lack joints, is that like Wnts and noggin, *Ihh* stimulates the target repressor gene Gli3 in interzone cells to delay chondrogenesis and permit joint formation (14). Conditional ablation of Gli3 or the *Ihh* receptor, *smoothened,* in the interzone should prove or disprove whether *Ihh* has this apparently counterintuitive anti-chondrogenic action.

The next step in which the interzone cavitates to form the joint may initially involve a fine line of apoptotic cells that preempts tissue loosening aided by motion. Hyaluronic acid is then secreted that interacts with its cognate receptor, CD44, resulting in the loss of tissue integrity and joint separation, functions that are assisted by the mucin-rich protein lubricin. Following cavitation, the two sides of a joint are shaped into reciprocal interlocking structures (see Figure 1). What remains arcane, however, is the accuracy underlying the genesis of a perfectly shaped humeral head or a patella that fits exactly over the knee.

4. ENDOCHONDRAL BONE FORMATION

The skeleton enlarges and ossifies

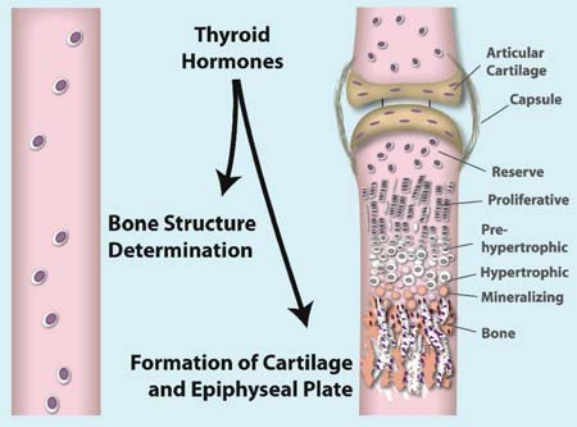

Figure 1. Effect of thyroid hormone on skeletal morphogenesis and growth exerted through chondrocyte thyroid hormone receptors.

mostly through the process of endochondral bone formation, and in flat bones like the skull, through intramembranous ossification. These processes require a set of non-redundant mechanisms that are spatially and temporally integrated. The loss of any one mechanism results in abnormal skeletal development, and in humans, a characteristic chondro-osseous dysplasia.

Endochondral bone formation begins with the condensation of mesenchymal cells and their transformation into prechondrocytes. Critical to lineage commitment is the transcription factor *Sox9*, which cooperates with the homeobox gene, *Barx-2 (*15). The transcription regulators *Pax1* and *Pax9,* as well as *Nkx3.1* and *Nkx3.3* regulate *Sox9* under the stewardship of *Shh (*16). These prechondrocytic cells then commit to form early chondroblasts, which undergo intense proliferation and secrete abundant aggrecan and type II collagen. To regulate this step, *Sox9* requires downstream partners *Sox5* and *Sox6,* the trio being both necessary and sufficient. Thus, while *Sox9*$^{-/-}$ and *Sox5*$^{-/-}$/*Sox6*$^{-/-}$ mice display reduced chondrocyte proliferation, impaired lineage commitment is seen only in *Sox9*$^{-/-}$ mice (17). A somewhat poorly understood step ensues next, in which centrally located chondrocytes stop proliferating and become post-mitotic prehypertrophic chondrocytes that secrete a collagen type X-rich matrix. The rounded cells closest to the hypertrophic zone flatten out into parallel longitudinal columns that continue to proliferate at rates that are highest away from the center. The proliferating cells also begin to express PTHrP. Both *Sox5* and *Sox6* are necessary for this conversion, as attested by a loss of cell columns in the *Sox5*$^{-/-}$/*Sox6*$^{-/-}$ mutants (18).

A critical phenotypic switch next marks the conversion of columnar and round chondrocytes into prehypertrophic and hypertrophic chondrocytes, both of which express high levels of collagen type IIb and aggrecan. The *Sox* trio delays hypertrophy at the time when the Runt family transcription factors *Runx2* and *Runx3* start being expressed. *Runx2* is negatively regulated by HDAC4, a histone deacetylase, but cooperates with the two distal-less-related homeobox transcription activators *Dlx5* and *Dlx6 (*19,20). *Runx2* is critical for chondrocyte hypertrophy and activation of collagen X, PTHrP receptor and *Ihh* gene transcription. Not unexpectedly, *Runx2*$^{-/-}$ mice and *Runx2*$^{-/-}$/*Runx3*$^{-/-}$ mutants lack prehypertrophic and hypertrophic chondrocytes.

Ihh and PTHrP interact in an elegant feedback in which *Ihh* secreted by prehypertrophic chondrocytes stimulates PTHrP release from periarticular cells. The released PTHrP maintains chondrocyte proliferation and prevents hypertrophy. *Ihh* also enhances chondrocyte proliferation directly and converts round chondrocytes to flat columnar cells (21,22). This positive feedback by *Ihh* ensures a constant flow of chondrocytes in and out of the columnar zone allowing for a timed and exactingly accurate linear expansion at the growth plate. Ablation of PTHrP or *Ihh* signaling disrupts this endochondral sequence producing severe runting and few or no columnar cells, a phenocopy of fetuses with Blomstrand's chondro-osteodystrophy. Conversely, mice over-expressing PTHrP or the constitutively active PTH/PTHrP receptor accumulate proliferating chondrocytes, an abnormality phenocopied in Jaansen's chondrodysplasia arising from a gain-of-function PTH/PTHrP receptor mutation.

Lateral signals from the surrounding perichondrium, namely BMPs, Wnts and FGF-2, regulate *Ihh* signaling (23). For example, *Ihh* enhances the expression of BMPs -2, -4 and -7, which in turn up-regulate Wnt-2a; these molecules work synergistically with *Ihh* to prevent hypertrophic differentiation. BMP-2 also down regulates Wnt-7a to achieve the same goal. TGFβ, cleaved from its precursor by metalloproteinases including MMP-13 also mediates *Ihh* action in inducing PTHrP expression (24). FGF signaling, in contrast, suppresses *Ihh* expression, reduces chondrocyte proliferation and enhances hypertrophy.

The differential coupling of the PTH/PTHrP receptor to $G_{s\alpha}$ or G_q exerts a second level of control. The targeted disruption of the $G_{s\alpha}$ signal causes chondrocyte hypertrophy, while that of G_q surprisingly permits it (25,26). These opposing actions nonetheless appear consistent with the concentration of PTHrP required for PTH/PTHrP receptor coupling with the respective G proteins (27). That lower PTHrP concentrations are required for $G_{s\alpha}$ coupling makes biological sense as such a coupling, most distant from the PTHrP source, will keep chondrocytes proliferating (27). Inhibition of p57, a cyclin-dependent kinase inhibitor, constitutes the proliferation signal (28), while *Sox9* activation prevents hypertrophy (29). Thus, the abrogation of p57 in PTHrP$^{-/-}$/p57$^{-/-}$ mice rescues, in part, the PTHrP null phenotype (28).

Closure of the chondrocyte differentiation program is initiated when hypertrophic chondrocytes become terminal chondrocytes and lose type X collagen. The transcriptional regulator c-*Maf* of the leucine zipper family finally mediates their apoptosis (30). Prior to apoptosis, a typical osteoblast gene sequence comprising MMP-13, alkaline phosphatase and osteopontin is initiated.

However, it is not clear why the classic osteoblastic gene collagen 1 remains silent in chondrocytes.

Osteogenesis is initiated when prehypertrophic and hypertrophic chondrocytes instruct perichondrial cells to become mineralizing osteoblasts. For this, *Ihh* signals through the receptor protein *smoothened* to induce BMP-2, BMP-4 and BMP–7, which then trigger the formation of a bone collar surrounding a primary ossification center. Thus, both s*moothened*$^{-/-}$ and compound PTHrP receptor/*Ihh* mice fail to ossify the perichondrium (31). *Runx2* expression in the osteoblast, a consequence of the down-regulation of its two repressors, *Twist-1* and *Twist–2,* coincides with its expression in hypertrophic chondrocytes. *Twist* deletion rescues certain, but not all, osteogenic defects in *Runx2*$^{-/-}$ mice (32). *Runx2,* a pre-requisite for osteogenesis, up-regulates a gene program consisting of collagen 1, osteocalcin, osteopontin, osteonectin and MMP-13.

A necessary late-onset player, the blood vessel, invades the perichondrium and penetrates the hypertrophic zone. This allows the removal of terminal chondrocytes and the procurement of perichondrial osteogenic cells that deposit the first traces of true bone, the primary spongiosa. Osteoclasts also enter through this ready vascular route to assist in the formation of a medullary cavity. They release proteolytic enzymes, such as MMP-9, which permit further vascular ingress. Vascularization is initiated by vascular endothelial growth factor (VEGF) that is secreted from hypertrophic chondrocytes and perichondrial cells in a *Runx2*-regulated manner. Interestingly, targeted VEGF ablation not only impairs vascularization and ossification allowing hypertrophic chondrocytes to accumulate, but also causes massive apoptosis. This pro-survival function of VEGF has been attributed to its epiphysis-specific isoform 120 (33,34).

Despite vascular penetration, the central portion of the growth plate remains relatively hypoxic. Hypoxic cells up-regulate HIF-1α, a transcription factor of the basic helix-loop-helix family that is normally ubiquitylated by the von Hippel Landau (VHL) tumor suppressor protein. VHL null mice thus show a striking decrease in chondrocyte proliferation that is normally seen when HIF-1α levels are upregulated by hypoxia. Additionally, conditional ablation of HIF-1α reveals abundant apoptotic chondrocytes in the central growth plate core; this suggests that HIF-1α is also required for the survival of hypoxic chondrocytes, likely through its known actions on glycolytic enzymes, such as phosphoglycerokinase (35).

The zinc-dependent proteases MMP-9, MMP-13 and membrane type-1 MMP (MT1-MMP) finally model and remodel the developing cartilage and bone. Gene ablation has defined functions for MMP-9 in chondrocyte removal, growth plate angiogenesis and osteoclast recruitment, and together with MMP-13, in ossification center and bone marrow cavity formation. Compound MMP-13-/-/MMP-9-/- mutants thus display persisting hypertrophic cartilage, reduced trabecular bone and delayed marrow cavity formation (36). Likewise, MT1-MMP-/- mice have severe defects in both endochondral and intramembranous ossification (37). Other MMPs, and their inhibitors, TIMPs, have been localized to distinct bone cell populations, but their true function, if any, remains unknown. The entire endochondral sequence (chondrocyte proliferation□chondrocyte hypertrophy□ vasculogenesis□osteogenesis) is recapitulated at the epiphysis to result in secondary ossification centers. A growth plate is thus created between primary and secondary ossification centers, a site at which longitudinal growth occurs up to puberty. In addition, appositional growth to further strengthen bone ensues when new bone is deposited beneath the periosteal membrane. Osteoclasts, derived from marrow cells, resorb bone on the endosteal surface. The two processes, sub-periosteal bone apposition and endosteal bone resorption, together determine the eventual thickness of long bones.

5. BONE FORMATION

Osteoblasts arise from stromal cell precursors in bone marrow to serve several distinct roles in post-natal life. They form new bone, regulate the genesis and resorptive activity of osteoclasts, control the egress of hematopoetic stem cells from bone marrow *niches,* and, in the guise of osteocytes, transduce mechanical stimuli. To form new bone, osteoblasts secrete type 1 collagen and non-collagenous proteins, including osteocalcin, osteopontin, osteonectin, bone sialoprotein and dentine matrix protein-1 (DMP-1), among others (38). These proteins regulate the content and character of the deposited mineral. Thus, mineral content increases in both osteonectin and osteopontin null mice (39-40), and decreases in DMP-1$^{-/-}$ mice (41), testifying to the negative and positive regulatory functions of the respective proteins.

Evidence that osteoblast differentiation and bone formation are governed by *Runx2* rests on the absence of bone formation in *Runx2*$^{-/-}$ mice; cleidocranial dysplasia in humans even with *Runx2* haploinsufficiency; and recent evidence for high bone mass *Runx2* polymorphisms (42). As a master switch, *Runx* prevents the differentiation of pluripotent stromal cells to lineages other than the osteoblast. Binding partners permissive to osteoblast differentiation include AP1 proteins, the Smads (Smad1 and 5), glucocorticoid and androgen receptors, several C/EBPs, Oct-1, *Dlx5*, Menin and Hes1. Those that inhibit *Runx2* activation include C/EBPδ, *Dlx3, Lef1, Msx2,* PPARγ, Smad3, Stat1 and *Twist.* In addition, various co-activators, such as p300, CBP, MOZ and MORF, and co-repressors such as HDACs 3, 4, 6, and a mediator of c-*src/yes* signaling YAP, modify *Runx2* activation.

This molecular assortment allows *Runx2* to serve as a platform for various cytokine and hormonal modifiers of osteoblast maturation. For example, BMPs, including TGFβ, bind to phosphorylated Smads to activate *Runx2* indirectly. PTH and the FGFs directly phosphorylate the Ser residue or the C-terminus, respectively. In contrast, TNF induces *Runx2* degradation *via* the E3 ubiquitin ligases smurf1 and smurf2 (43). What is fascinating, however, is that *Runx2* delimits these multiple signal

cascades to achieve remarkable temporal homogeny in inducing its osteogenic gene program. It has been suggested that *Runx2* binding occurs in discrete nuclear matrix compartments, and that chromatin remodeling modifies these spatial domains to specify gene expression sequences during osteoblast differentiation.

Like *Runx*$^{-/-}$ mice, mice deficient in another transcription factor, *osterix* do not form bone. Nonetheless, while *Runx2*$^{-/-}$ mice lack *osterix*, the reverse is not true, suggesting that *osterix* is downstream of *Runx2*. Like *Runx2, osterix* expression is regulated by BMP-2, IGF-1 and TNF. Importantly, however, to mediate collagen 1 expression, *osterix* must form a complex with the NFAT transcription factor family member NFAT2 (44). Bone formation is therefore reduced dramatically in mice deficient in the phosphatase, calcineurin that activates NFAT2. Over-expression of calcineurin enhances osteoblast differentiation and bone formation (45). In contrast to *osterix*, a CREB-related transcription factor, ATF4, is the substrate for a growth factor-regulated kinase, RSK2, a mutation in which causes the skeletal defects in Coffin-Lowry syndrome (46). Ablation of Rsk2 or ATF4 delays bone formation, indicating an effect on the terminal differentiation of osteoblasts. ATF4 transactivation is inhibited by FIAT, a leucine-zipper nuclear protein (47).

Evidence that Wnts regulate osteoblastogenesis comes from the osteopenia and impaired osteoblast proliferation noted either upon the ablation of Wnt co-receptors, *Lrp5* and *Lrp6*, or the use of the Wnt inhibitors, such as sclerostin, soluble frizzled-related protein (sFRP) 1-3, Wnt inhibitory factor-1 *(Wif-1)* and the *Dickkoff* family members *Dkk1* and *2 (*48-51). Likewise, in humans, loss- and gain-of-function mutations of *Lrp5* cause osteoporosis pseudoglioma syndrome and a high bone mass phenotype (52), respectively. Canonical Wnt signaling stabilizes and permits the nuclear ingress of β-catenin to stimulate osteoblast differentiation cooperatively with the *Tcf/Lef* transcription factors. Thus, Wnt10b induces *Runx2*, *Dlx5* and *osterix* and suppresses PPARγ to shift stromal cell differentiation towards the osteoblastic lineage (53). Wnt 3a and 5a additionally prevent osteoblast apoptosis (54). In conjunction with the early B-cell factor EBF2, Wnts also regulate osteoclast formation by altering the production of osteoprotegerin, the decoy receptor for RANK-L (55,56). Thus, β-catenin or EBF2 null mice have osteopenia, striking increases in osteoclasts, and reduced osteoprotegerin levels (56).

That the osteoblast is a key regulatory component of the hematopoetic stem cell *niche* has only been established recently (57,58). Osteoblastic over-expression of the constitutively activated PTH/PTHrP receptor causes a focal increase in the expression of a *Notch* ligand, *jagged-1,* as well as increased stem cell numbers, but importantly, within these cells, the activation of *Notch* signaling. The latter result unquestionably confirms proximity between hematopoetic stem cells and osteoblasts (58). The conditional ablation of osteoblasts likewise causes substantial decreases in stem cell numbers (59). Impressive, however, has been evidence that stem cell egress from the *niche* is regulated by sympathetic nerve signals directed to the osteoblast (60). Demyelination in ceramide galactosyltransferase-null mice and, more specifically, abrogation of norepinephrine release prevents the forced egress of hematopoetic stem cells from the *niche* in response to GM-CSF (60). This provides the first functional evidence that osteoblasts and stem cells talk within a *niche.*

Once mineral is deposited, an osteoblast becomes buried within its own matrix to become an osteocyte, which, *albeit* encased, communicates with neighboring osteocytes through a dense canalicular network. Osteocytes sense and transduce changes in fluid flow arising from stress, strain or pressure. The outcome of skeletal loading is unequivocal: even brief loading periods elicit new bone formation in almost every species. Nonetheless, the molecular identity of the putative mechanosensing receptor has remained a mystery. Proposed candidates include L-type voltage-gated Ca^{2+} channels, β_1 integrin, connexins and lipid rafts, as well as the possibility that altered cell shape is sensed directly (61). Loading also induces canalicular hypoxia that up-regulates Hif-1α (62). While downstream events involve the MEK/Erk and IP_3/Ca^{2+} pathways, there is little information on how these signals are integrated in space and time.

6. BONE RESORPTION

The first evidence for the hematopoetic origin of osteoclasts came from parabiosis experiments and spleen cell transplantation. Since then, osteoclasts, macrophages, dendritic cells and lymphocytes have been shown to share molecules and mechanisms for their differentiation. The myeloid and B cell transcription factor PU.1 first determines the osteoclast lineage. Macrophage colony stimulating factor (M-CSF) next ensures the proliferation and survival of committed precursors. The T-cell factor RANK-L then diverts these precursors away from the macrophage toward the osteoclast lineage. Lymph node development is similar in that, like osteoclast formation, it also involves hematopoetic/ stromal cell, integrin/VCAM and RANK/RANK-L interactions. RANK-L$^{-/-}$ mice thus lack osteoclasts and display impaired lymph node maturation.

The action of RANK-L on the osteoclast precursor involves interacting kinase and transcription factor cascades. A critical event is the recruitment of the docking protein TRAF-6 to the cytoplasmic domain of the RANK receptor. TRAF-6 mediates NF-κB and MAP kinase activation. Cytosolic Ca^{2+} oscillations arise in parallel through phospholipase Cγ activation by *Syk* kinases that are recruited to the ITAM-harboring adapters DAP12 and FcRγ (63). That both *Syk*$^{-/-}$ and DAP12$^{-/-}$/FcRγ$^{-/-}$ mice have osteopetrosis suggests an obligatory role for ITAM co-stimulation in osteoclastogenesis (64). Upstream in this pathway are TREM2 and OSCAR, recently discovered receptors in search of ligands (64). Downstream, the Ca^{2+} oscillations trigger the phosphatase calcineurin to activate the transcription factor NFAT2. NFAT2 is not only indispensable, but is sufficient for osteoclast formation.

Thus, embryonic precursors from NFAT2$^{-/-}$ mice fail to become osteoclasts and over-expression of NFAT2 yields osteoclasts even without RANK-L (65-67). In addition, NFAT2 stimulates its own expression as well as that of OSCAR (68,69). Negative feedback, in contrast, results from the induction of *jun* dimerization protein (JDP-2) that blocks AP-1-dependent gene transcription (70).

For the resorption of bone, an osteoclast creates a highly acidic compartment beneath itself, within which its membrane is thrown into complex folds, the ruffled border that harbors all secretory activity. Formation of this sealed specialization, *akin* to a phagolysosome, requires the precise control of adhesion, motility and polarization. For adhesion, matrix RGD peptides trigger the integrin $\alpha_v\beta_3$ to recruit c-*src* that interacts with another kinase *Syk;* this interaction is regulated by the guanine nucleotide-binding factor *Vav-3* (71). Adhesion additionally triggers membrane ruffling and granule extrusion, processes that require the minute-to-minute assembly and disassembly of actin. For this, $\alpha_v\beta_3$ activates c-*src* and *Pyk-2;* c-*src* then recruits the multi-site adapters c-*Cbl* and *Cbl*-b, which complex with phosphotidylinositol 3-kinase (PI-3kinase) and a GTPase, dynamin (72). Several mechanistic uncertainties exist. First, while *src* is essential for polarization, we are not confident that its kinase activity is imperative. Second, we are not clear if complexes between gelsolin or cortactin and the integrin-associated proteins paxillin, talin and vinculin also contribute. Third, we can only speculate how the diverse array of proteins that include scaffolds, actin-associated VASPs, ITAM-harboring proteins and *src* and *syk* adapters delimit and integrate higher signals towards specific outcomes.

A multi-subunit V-type H^+-ATPase pumps H^+ across the ruffled border causing the ambient pH to fall to <4U. This allows acid-optimal enzymes, such as cathepsin K, to cleave the helical and telopeptide regions of collagen and release peptides that are transcytosed to exit at the dorsolateral surface. A Cl^- countercurrent through ClC-7 balances proton extrusion and an HCO_3^-/Cl^- exchanger corrects any cellular alkalinization. That H^+-ATPase, C1C-7 and cathepsin K are obligatory to resorption is attested by the profound osteopetroses resulting from their deficiency. The low pH also causes hydroxyapatite dissolution that elevates ambient Ca^{2+} to around 40 mM. This activates a putative Ca^{2+} sensor allowing the cell to detach and retract. The pro-resorptive cytokine interleukin-6 is then released in a feedback loop to inhibit further Ca^{2+} sensing and reestablish resorption (73). Despite our own efforts to affirmatively establish its identity, the osteoclast Ca^{2+} sensor still remains a putative entity (73). Homology cloning has largely failed to identify a member of the Ca^{2+} sensing receptor (CaSR) family in osteoclasts. Furthermore, CaSR deficiency in mice or humans does not cause osteoclast defects. The cation channel, TrypV5, has been implicated, but its deletion results in inactive rather than hyperactive osteoclasts (74). We find that ryanodine receptor-II (RyR-II) of the endoplasmic reticular Ca^{2+} channel family is located in the osteoclast plasma membrane (75). In addition to its role as a Ca^{2+} influx channel, RYR-II likely functions as the Ca^{2+} sensor with its low affinity Ca^{2+}-binding site facing outwards (76). Attesting to this view is the elevated resorption seen in CD38$^{-/-}$ mice, in which the levels of cyclic ADP-ribose, a physiologic RyR agonist, are reduced (77).

Signals that initiate osteoclast apoptosis following multiple episodes of resorption are unknown. In contrast, survival signals such as $\alpha_v\beta_3$ and M-CSF are required to ensure the maintenance of osteoclast precursors. Thus, removing negative regulators of M-CSF, such as SHIP, results in abundant osteoclasts (78). Key to the anti-apoptotic effect of M-CSF is the inactivation of the pro-apoptotic gene *Bim* through *Cbl*-mediated ubiquitylation. Although the loss of *Bim* prevents apoptosis, surviving osteoclasts are surprisingly less active (79). This peculiar phenotype possibly arises from the conflicting role of c-*Cbl* acting upstream, as both a c-*src* anchor during resorption and ubiquitin ligase during apoptosis. This may be why c-*Cbl*$^{-/-}$ mice do not display an overt phenotype.

7. NEUROGENIC CONTROL OF BONE MASS

Karsenty's discovery that central leptin inhibits bone formation, and importantly, that the sympathetic nervous system mediates this effect adds a novel dimension to bone physiology[80,81]. Intracerebroventricular injection of leptin causes profound bone loss, whereas the disruption of leptin signaling increases bone mass despite accompanying hypogonadism and hypercortisolism (80). The effect of central leptin is lost with the ablation of the adrenergic receptor *Adrb*2 or dopamine β-hydroxylase *(Dbh)*. β-adrenergic drugs therefore expectedly affect bone mass (81). However, they do not affect body mass, suggesting that bone mass and body mass control by central leptin occurs through distinct mechanisms[81]. Furthermore, high bone mass in the *Ardb2*$^{-/-}$ mouse arises not only from enhanced bone formation, but also from low bone resorption. Sympathetic signaling through ATF4 up-regulates RANK-L to increase resorption; this effect is inhibited by CART (cocaine amphetamine-related transcript), a leptin-regulated neuropeptide (82). Additionally, by interacting with the clock genes *Per* and *Cry* in the osteoblast, adrenergic stimulation provides circadian rhythmicity. Mice lacking *Per* and *Cry*, or *Per* in osteoblasts, have high bone mass. Finally, in contrast to leptinergic control, evidence for the role of peptidergic neurons in bone mass regulation is limited. While the targeted ablation of NPY2 receptors increases bone mass (83), NPY$^{-/-}$ mice lack bone defects (84). Likewise, mice without CGRPα have low bone formation (85), but it unclear whether the effect is central or peripheral.

8. ENDOCRINE REGULATION OF BONE MASS

The main, if not exclusive, hormone regulating bone formation is IGF-1, secreted in response to growth hormone (86). IGF-1$^{-/-}$ mice and compound IGF-1/IGF-1 receptor null mice show profound runting postnatally (87), while IGF-2$^{-/-}$ mice are retarded only *in utero* but grow normally after birth. There is strong epidemiological and genetic evidence for correlations between serum IGF-1 and bone mass in humans and mice.

Table 1. The association of various thyroid disorders and modalities of thyroid hormone therapies on bone loss and fracture risk[1]

Scenario	Markers	Clinical Correlates	Ref
Primary hypothyroidism		↑ fracture risk	112-114
Subclinical hyperthyroidism		↑ fracture risk	112
TSH suppression therapy			
Benign disease		↑ fracture risk	112
Thyroid cancer	↑ cross-links	ns to ↓ spine or hip BMD up to 5-9%	104-107
Thyroid hormone replacement	↑ cross-links	ns to ↓ spine BMD by 5%	108-110
Normal TSH		ns to ↓ hip BMD by <1 to 7%	108-111
		No effect on fracture risk	112,114

[1]only longitudinal studies included; ns – not statistically different

Nonetheless, the ablation of liver IGF-1 or the acid labile subunit, which prevents IGF-1 degradation, together provide incontrovertible evidence that circulating rather than tissue IGF-1 regulates bone mass (88). These studies also show that the elevated growth hormone levels do not compensate for circulating IGF-1 deficiency.

Hormones that inhibit bone resorption, namely estrogen, calcitonin and TSH do so through direct osteoclastic actions. In contrast, most pro-resorptive stimuli including PTH and 1,25-dihydroxyvitamin D_3 first stimulate the osteoblast, which then secretes RANK-L. The exception is FSH. Unlike calcitonin, estrogen does not inhibit bone resorption by mature osteoclasts. Instead, it attenuates osteoclast formation by reducing JNK/AP-1 activation directly; by decreasing stromal cell cytokine release indirectly; and *via* T lymphocytes by reducing TNF and increasing interferon-γ production (89). Estrogen also prevents osteoblast apoptosis through non-genomic actions of unclear relevance (90). Nonetheless, despite being used widely for osteoporosis therapy, we remain uncertain which of these mechanisms are of physiologic importance. In contrast, the exquisite sensitivity of an osteoclast to calcitonin arises from over a million receptors per cell that couple to either G_s or G_q (91). Despite this mechanistic clarity, we remain uncertain if calcitonin is a true *in vivo* regulator of bone resorption. This is because deletion of calcitonin and its alternate splice product, CGRP, or the calcitonin receptor (CTR) yields unexpected high bone mass rather than osteopenic phenotypes (91-92). In contrast, mice deficient in the related peptide amylin display osteopenia. However, it is unlikely that amylin interacts with the CTR as compound amylin$^{-/-}$/CTR$^{-/-}$ mice have a dual phenotype (93).

We have recently reported novel actions of the anterior pituitary hormones TSH and FSH on bone resorption. TSH reduces osteoclast formation, function and survival consistent with the osteopenic phenotype in TSH receptor (TSHR) haploinsufficient mice (94) (this is discussed in greater detail in the next section). In contrast, FSH stimulates osteoclast formation and function congruent with the high bone mass phenotype of FSHβ haploinsufficient mice (95). TSH and FSH oppositely affect NF-κB activation, whereas TSH additionally inhibits JNK/c-*jun* and FSH stimulates both *Erk* and Akt *via* $G_{i2\alpha}$.

9. BONE LOSS FROM ALTERATIONS IN THE PITUITARY OR THYROID AXIS

The osteoporosis of hyperthyroidism has similarly been attributed to high thyroxine levels. However, TSHR haploinsufficient mice are osteopenic with enhanced osteoclastogenesis, despite normal follicular structure and serum thyroxine (94). Supplemented euthyroid patients with TSH receptor mutations likewise experience significant high-turnover bone loss (96). More impressive, however, is the tight correlation between serum TSH levels and fracture risk in hyperthyroid patients, as well as recent evidence for the direct suppression of bone remodeling by recombinant TSH in post-menopausal women (97,98). Together, the evidence suggests that TSH regulates bone mass, and that its deficiency causes bone loss.

Thyroid dysfunction and thyroid hormone therapy have been associated with derangements in bone metabolism. Hyperthyroidism, a state characterized by elevated thyroid hormone levels and suppressed TSH levels, is associated with elevated bone turnover and ultimately decreased bone mass. In hyperthyroid patients each bone resorption cycle is shortened and excessive (99). Compounding the excessive bone degradation, absorption of intestinal calcium and phosphate is decreased, while dermal, fecal, and urinary calcium losses are increased; these changes produce a state of negative calcium balance that negatively impacts bone mass (100). This increased bone turnover in hyperthyroid states is evident in clinical markers of bone turnover; there are increases in bone-specific alkaline phosphatase, osteocalcin, carboxyterminal propeptide of type 1 collagen (P1NP), and carboxy- and N-terminal cross-linked telopeptides of type 1 collagen (CTx and NTx, respectively) (101-103).

Ten years ago, the prevailing paradigm was that thyroid hormones (T3/T4) directly impacted bone metabolism. This notion was supported by numerous longitudinal studies demonstrating associations between the thyroid state and bone status (Table 1) (104-114). In laboratory studies, thyroid hormones were shown to directly activate bone resorption through the nuclear thyroid hormone receptors (TR) α and β (115). Further studies served to elucidate that thyroid hormone-increased osteoclastic resorptive activity occurred indirectly through osteoblasts and fibroblast growth factor receptor-1

Table 2. Skeletal effects of thyroid hormone receptor ablation

Mouse Model	Phenotype (123)	Phenotype (116)
$TR\alpha^{-/-}$	Defects in skeletal maturation	Defects in skeletal maturation and high bone mass
$TR\beta^{-/-}$	Defects in skeletal maturation	Defects in skeletal maturation and osteopenia
$TR\alpha^{-/-}\beta^{-/-}$	Defects in skeletal maturation	Not tested

(FGFR1); various inflammatory cytokines and other hormones were implicated in thyroid hormone-induced bone loss, including IL-6, PGE2, PTH, and 1,25-dihydroxyvitamin D (116-117).

Thus, thyroid hormones are intertwined amongst the cytokines and hormones regulating bone metabolism. However, with the analysis of thyroid hormone receptor knockouts, it became apparent that the function of thyroid hormones was not to regulate bone metabolism but rather to regulate bone morphogenesis and development.

In bone, the thyroid hormone receptor TRα is expressed at higher levels than TRβ and has thus been thought to be functionally predominant (118-119). $TR\alpha^{-/-}$ mice, which lack TRα, but are biochemically euthyroid because of compensation through TRβ, have defects in skeletal morphogenesis (120) (see Table 2). On the other hand, $TR\beta^{-/-}$ mice, which lack TRβ isoforms and have thyroid hormone resistance, do not have defects in skeletal morphogenesis (121-122). $TR\alpha^{-/-}\beta^{-/-}$ mice do not develop any bone remodeling defects, but rather, manifest skeletal maturation defects with runting and growth plate abnormalities (123).

Despite evidence linking thyroid hormones to bone morphogenesis and skeletal development, clinically, the adverse effects of thyrotoxicosis is due to excess bone resorption (114,124-131). This incongruence prompted us to challenge the notion that thyroid hormone was the key hormone responsible for altering bone metabolism. In 2003, we discovered that thyroid-stimulating hormone (TSH; thyrotropin) can directly regulate bone remodeling (132). We generated euthyroid mice heterozygous for a deletion of the TSH receptor (TSHR) gene and found that these animals had reduced BMD and increased markers of bone turnover (133). Similarly, homozygous $TSHR^{-/-}$ mice made euthyroid through repletion with levo-thyroxine also had a reduced BMD (132). These two animal models suggested that derangement of the TSH levels could affect bone metabolism despite a normal thyroid axis. On characterizing the *in vivo* bone phenotype of these animals, we found that they had high-turnover osteoporosis with focal osteosclerosis, which is typically associated with uncoupling between bone formation and resorption (132). *Ex vivo* studies indicated that TSH affects osteoclast and osteoblast function and formation via multiple mechanisms (see Figure 2). Initially, we found that TSH increased osteoclast apoptosis, and decreased osteoblast differentiation and collagen synthesis (132-134). RANKL-induced osteoclastogenesis is inhibited by TSH at two signal transduction steps involving inhibitor κBα (IκBα) and JNK, which in turn lead to depressed nuclear levels of the pro-osteoclastogenic transcription factors c-jun and p65 (132,135,136). The depressed c-jun levels impair tartrate-resistant acidic phosphatase (TRAP) and cathepsin K gene transcription, which are critical to osteoclastic bone resorption (137).

Further experiments defined TNFα as the critical cytokine mediates the downstream effects of TSH (138). Specifically, TSH inhibits cytokine-induced TNFα production in the bone marrow microenvironment (138). Crossing the $TSHR^{-/-}$ animals to $TNF\alpha^{-/-}$ animals showed that the effect of TSH on bone metabolism could be reversed, thus providing *in vivo* evidence linking TSH and TNFα (138-139).

10. CLINICAL CORRELATIONS AND RECOMMENDATIONS

In line with the discovery that TSH can directly regulate bone metabolism in a thyroid hormone-independent manner, a 2004 review of population-based studies by Murphy and Williams concluded that endogenous and exogenous TSH suppression, but not thyroid hormone therapy *per se*, was associated with an increased fracture risk; this adverse effect did not necessarily correlate with decreased bone mineral density (BMD) (140). A high fracture risk in the face of a normal bone density is not surprising and is usually an accompaniment of high dose glucocorticoid therapy, immunosuppression and acute immobilization (141). Consistent with this, two cross-sectional studies, which did not report fracture risk, showed no adverse effect of thyroid hormone suppression therapy on biochemical or densitometric parameters of skeletal integrity (142-143).

Moreover, in subjects with TSH receptor mutations, but euthyroid due to l-thyroxine supplementation, there is decreased BMD also with elevated markers of bone remodeling, such as osteocalcin and N-telopeptide (see Table 1) (128). These physiological correlations are consistent with the clinical observations mentioned above, in which patients with suppressed TSH levels have decreased BMD and increased fracture risk.

Similar evidence also comes from an impressive epidemiological study by Bauer and colleagues, who used serum TSH levels to predict the risk of fracture in hyperthyroid post-menopausal women (144). Most impressive was the increase in vertebral and hip fracture risk by 4.5- and 3.3-fold, respectively, when serum TSH was <0.1 mIU/ml (144). No correlations between thyroid hormone levels and fracture risk were observed. Rather than being a causal relationship, this correlation could arise because TSH suppression is more sensitive index of thyroid

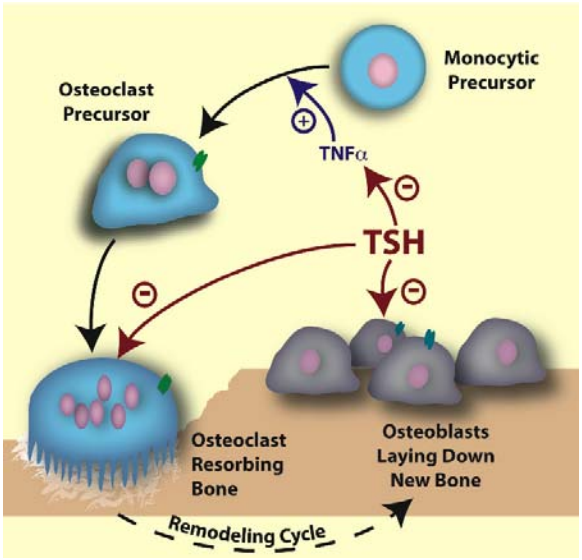

Figure 2. The effect of TSH on the two components of bone remodeling, osteoclastic bone resorption and osteoblastic bone formation. TSH inhibits both osteoblast differentiation and osteoclast formation; the latter action is exerted directly as well as indirectly via suppression of the cytokine TNFa, which normally induces osteoclast precursor proliferation.

hormone excess than thyroid hormone levels per se. However, subjects with TSH receptor mutations rendered euthyroid through thyroid hormone supplementation were found to display a high turnover osteoporosis (see Table 1) (128). Thus, in the light of genetic and pharmacological evidence for direct effects of TSH on bone, it is highly likely that a low TSH contributes to hyperthyroid bone loss.

Because of the strong correlation between low TSH levels and a high fracture risk, which appears to be dissociable from long-term decrements in bone mineral density, we suggest maintaining TSH levels during replacement therapy to above 1 mU/mL, unless there is a clinical rationale for TSH suppression as in thyroid cancer patients. In these patients, admittedly without clinical evidence of efficacy, we propose the empiric use of an oral bisphosphonate to prevent the high turnover osteoporosis and associated fracture risk, which appears to be highly correlated to a TSH level of <0.1 mU/mL.

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