Role of thyroid hormones in skeletal development and bone maintenance

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The skeleton is an exquisitely sensitive and archetypal T3-target tissue that demonstrates the critical role for thyroid hormones during development, linear growth, and adult bone turnover and maintenance. Thyrotoxicosis is an established cause of secondary osteoporosis, and abnormal thyroid hormone signaling has recently been identified as a novel risk factor for osteoarthritis. Skeletal phenotypes in genetically modified mice have faithfully reproduced genetic disorders in humans, revealing the complex physiological relationship between centrally regulated thyroid status and the peripheral actions of thyroid hormones. Studies in mutant mice also established the paradigm that T3 exerts anabolic actions during growth and catabolic effects on adult bone. Thus, the skeleton represents an ideal physiological system in which to characterize thyroid hormone transport, metabolism, and action during development, adulthood and in response to injury. Future analysis of T3 action in individual skeletal cell lineages will provide new insights into cell-specific molecular mechanisms and may ultimately identify novel therapeutic targets for chronic degenerative diseases such as osteoporosis and osteoarthritis. This review provides a comprehensive analysis of the current state-of-the-art.

I. Introduction

THE ESSENTIAL REQUIREMENT for thyroid hormones during linear growth and skeletal maturation is well established and has been recognized for 125 years. Indeed, the association between goiter, cretinism, developmental retardation and short stature had been known for centuries, and the therapeutic use of burnt sponge and seaweed in the treatment of goiter dates back to 1600 BC in China. Paracelcus provided the first clinical description of endemic goiter and congenital idiocy in 1603. Between 1811–1813 Bernard Courtois discovered iodine, Joseph Gay-Lussac identified it as an element and Humphrey Davy recognized it as a halogen (1). However Jean-Francois Coindet, in 1820, was the first to use iodine as a treatment for goiter and Gaspard Chatin, in the 1850's, was the first to show that iodine in plants prevented cretinism and goiter in endemic regions. Thomas Curling, in 1850, described cretinism in association with athyreosis, while William Gull provided the causal link between lack of a thyroid gland and cretinism in 1873. William Ord extended Gull's observations and chaired the first detailed report on hypothyroidism by the Clinical Society of London in 1878 linking cretinism, myxedema and cachexia strumipriva (decay due to lack of goiter) as a single entity. Indeed, in a lecture to the German Society of Surgery in 1883, the Swiss Nobel Laureate Theodor Kocher described cachexia strumipriva as a specific disease that included "decreased growth in height" following removal of the thyroid gland. Ultimately, these events led to the first organotherapy for hypothyroidism by George Murray in 1891, although the ancient Chinese had used animal thyroid tissue as a treatment for goiter as early as 643 AD (1–3).

Alongside the emergence of hypothyroidism as a recognized disease, Charles de Saint-Yves, Antonio Testa and Guiseppe Flajani reported the first cases of goiter, palpitations and exophthalmos between 1722–1802, although these features were not linked at that time. Caleb Parry had recognized in 1825, while Robert Graves independently recognized and also published in 1835, the link between hypertrophic goiter and exophthalmos (1, 3). Carl Adolf von Basedow extended Graves' description in 1840 by adding palpitations, weight loss, diarrhea, tremor, restlessness, perspiration, amenorrhea, myxedema of the lower leg and orbital tissue hypertrophy to describe the

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syndrome more completely. In 1886, Paul Möbius proposed the cause of these symptoms was increased thyroid function and Murray supported this view in 1891 at the time of his organotherapy for hypothyroidism (1, 3). Coincidentally, in the same year of 1891, Friedrich Von Recklinghausen reported a patient with thyrotoxicosis and multiple fractures and was the first to identify the relationship between the thyroid and the adult skeleton (4, 5). Since then a role for thyroid hormones in bone and mineral metabolism has become well established.

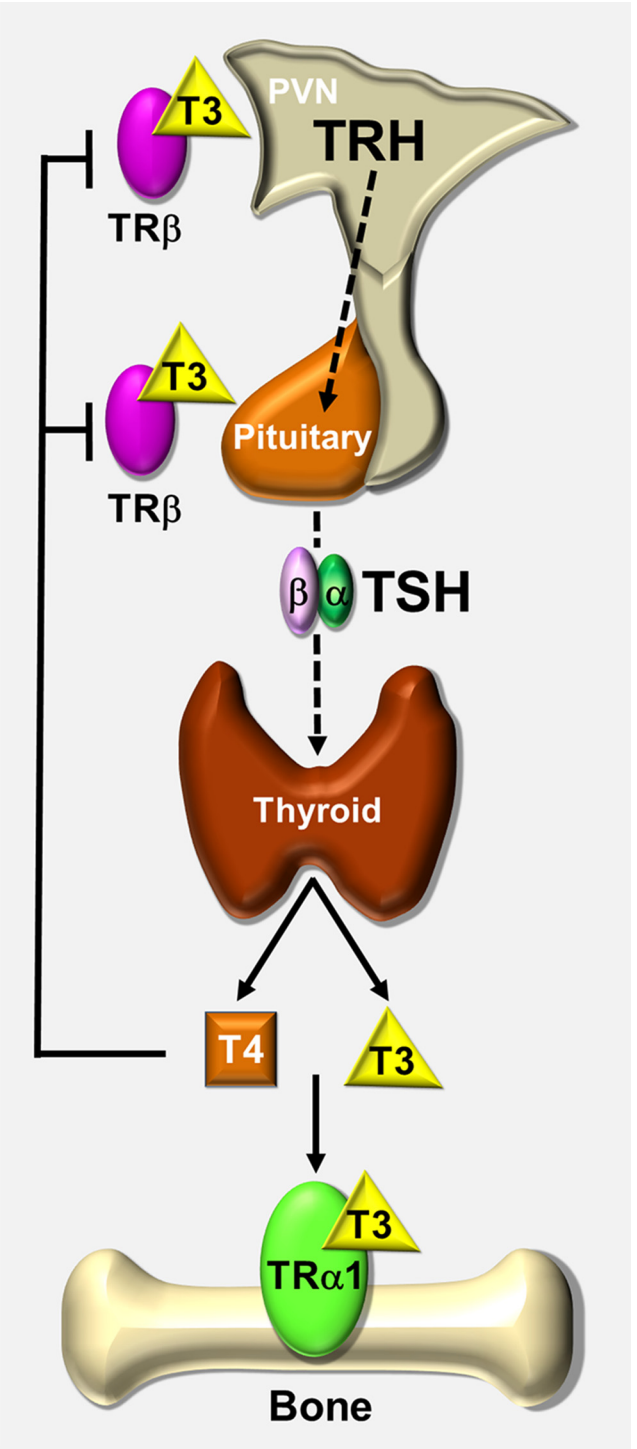
During the last 25 years the role of thyroid hormones in bone and cartilage biology has attracted considerable and growing attention, leading to important advances in understanding the consequences of thyroid disease on the developing and adult skeleton. Major progress in defining the mechanisms of thyroid hormone action in bone has followed and led to new insights into thyroid-related skeletal disorders. As a result, the role of the hypothalamic-pituitary-thyroid (HPT) axis in skeletal pathophysiology has become a high profile subject. It is only now that experimental tools are becoming available to allow determination of the precise cellular and molecular mechanisms that underlie thyroid hormone actions in the skeleton. This review will discuss our current understanding by considering the published literature up to December 31st 2015.

II. Thyroid hormone physiology

A. Hypothalamic-pituitary-thyroid axis

Synthesis and release of the prohormone 3,5,3′,5′-L-tetraiodothyronine (thyroxine, T4) and the active thyroid hormone 3,5,3′-L-triiodothyronine (T3) are controlled by a negative feedback loop mediated by the HPT axis (Figure 1) (6). Thyrotropin releasing hormone (TRH) is secreted by the hypothalamic paraventricular nucleus and acts on pituitary thyrotrophs to stimulate release of thyrotropin (thyroid-stimulating hormone, TSH). TSH subsequently acts via the TSH receptor (TSHR) on thyroid follicular cells to stimulate cell proliferation and the synthesis and secretion of T4 and T3 (7). T3, derived predominantly from local metabolism of T4, acts via thyroid hormone receptors α and β (TRα, TRβ) in the hypothalamus and pituitary to inhibit synthesis and secretion of TRH and TSH (8–11). Normal euthyroid status is maintained by a negative feedback loop that establishes a physiological inverse relationship between TSH and circulating T3 and T4, thus defining the HPT axis set point (12, 13). Systemic thyroid hormone and TSH concentrations vary significantly among individuals, indicating each person has a unique set point (12). Twin studies indicate the set point is genetically determined with heritability for free T3 (fT3), free T4 (fT4) and TSH of 65% (14), and candidate

Figure 1.



Hypothalamic-pituitary-thyroid axis

The thyroid gland secretes the prohormone T4 and the active hormone T3 and circulating concentrations are regulated by a classical endocrine negative feedback loop that maintains an inverse physiological relationship between TSH, and T4 and T3.

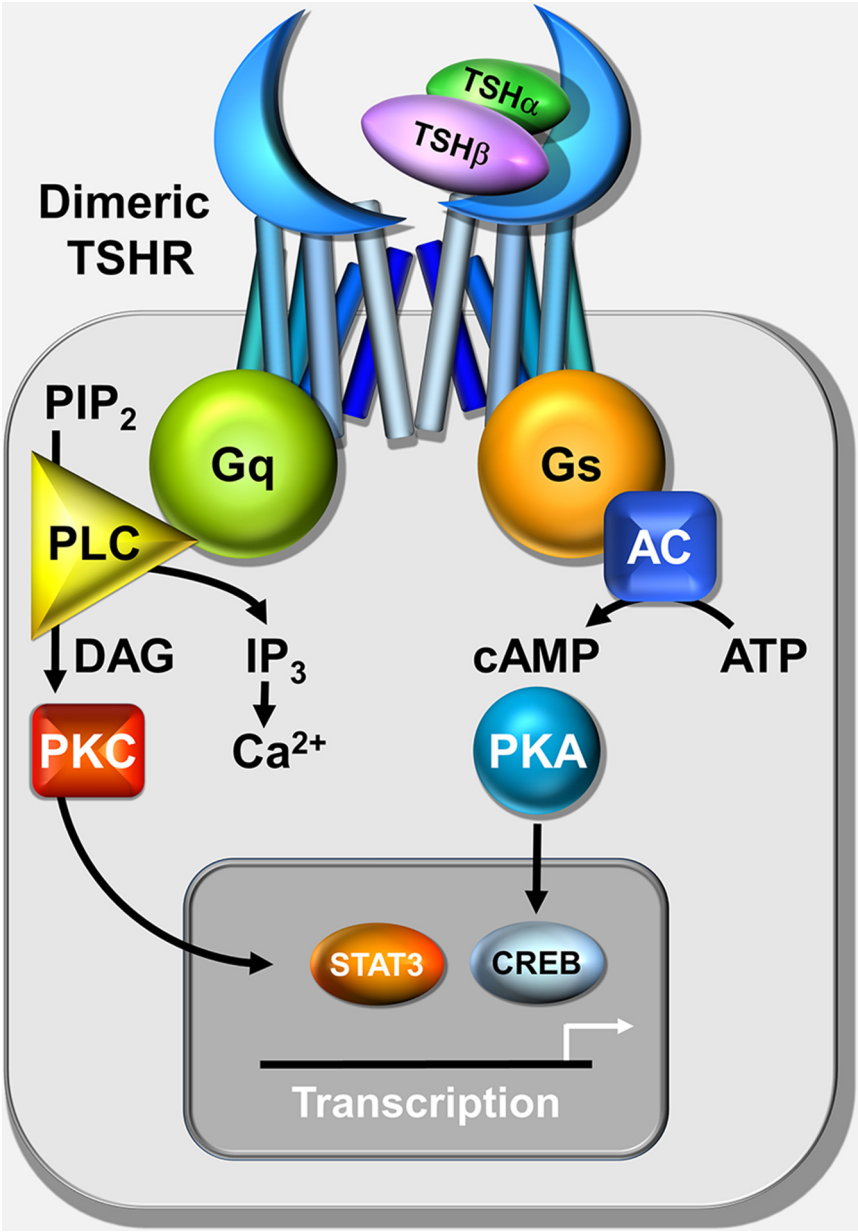
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gene and genome wide association studies (GWAS) have identified quantitative trait loci (15–17).

B. TSH action

The glycoprotein hormone TSH is composed of common α- and unique β-subunits. The TSHR is a G-protein coupled receptor consisting of ligand binding, ecto- and transmembrane domains (Figure 2) (18). Although cAMP is the major second messenger following activation of the TSHR in thyroid follicular cells, alternative downstream signaling pathways have been implicated in both thyroid and extrathyroidal tissues (19–22). In the thyroid the TSHR associates with various G proteins (23), and Gαs and Gαq are thought to compete for activation by the TSHR (20, 24).

Figure 2.



TSH action

Binding of TSH to the TSHR results in activation of G protein coupled downstream signaling including the (i) adenylyl cyclase (AC), cAMP, protein kinase A (PKA) and the cAMP response element binding (CREB) protein or (ii) phospholipase C (PLC), inositol triphosphate (IP_3) and intracellular calcium pathway or the (iii) PLC, diacylglycerol (DAG), protein kinase C (PKC), and signal transducer and activator of transcription 3 (STAT3) pathway.

C. Extrathyroidal actions of TSH

The TSHR has been proposed to have diverse functions in extrathyroidal tissues, although their physiological importance has not been established. Thus, TSHR expression has been reported in anterior pituitary, brain, pars tuberalis, bone, orbital preadipocytes and fibroblasts, kidney, ovary and testis, skin and hair follicles, heart, adipose tissue, as well as hematopoietic and immune cells (19, 25–28). These data suggest direct actions of TSH, for example, in the regulation of seasonal reproduction (29–31), bone turnover (32), pathogenesis of Graves' orbitopathy (33–35), and immunomodulatory responses in the bone marrow (36–43), gut (42, 43) and skeleton (38).

D. Thyroid hormone transport

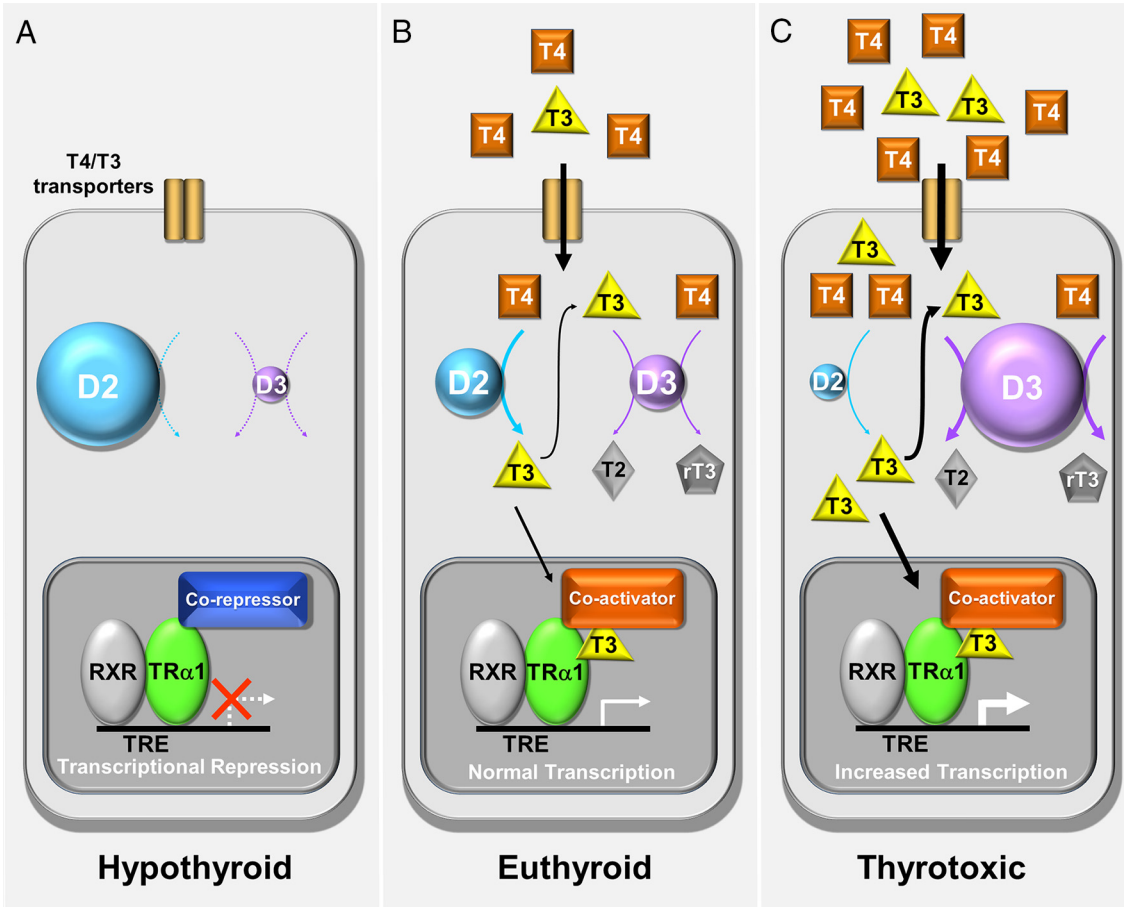
Uptake of thyroid hormones into peripheral tissues and entry into target cells is mediated by specific membrane transporter proteins (Figure 3) (44), including monocarboxylate transporters MCT8 and MCT10, the organic anion transporter protein-1C1 (OATP1C1), and the non-specific L-type amino acid transporters 1 and 2 (LAT1, LAT2) (45). The best-characterized specific transporter MCT8 is expressed widely and its physiological importance has been demonstrated by inactivating mutations of *MCT8* that cause the Allan–Herndon–Dudley X-linked psychomotor retardation syndrome (OMIM #300523) (46, 47).

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E. Thyroid hormone metabolism

T4 is derived from thyroid gland secretion, while most circulating T3 is generated by deiodination of T4 in peripheral tissues. Although the circulating fT4 concentration is fourfold greater than fT3, the TR-binding affinity for T3 is 15-fold higher than its affinity for T4 (48). Thus, T4 must be converted to T3 for mediation of genomic thyroid hormone action (Figure 3) (49). Three iodothyronine deiodinases metabolize thyroid hormones to active or inactive products (6, 50, 51). The type 1 deiodinase (DIO1) is inefficient with an apparent Michaelis constant (Km) of 10^{-6}-10^{-7} M, and catalyzes removal of inner or outer ring iodine atoms in equimolar proportions to generate T3, reverse T3 (rT3), or 3,3′-diiodothyronine (T2) depending on the substrate. Most of the circulating T3 is derived from conversion of T4 to T3 by DIO1, which is expressed mainly in the thyroid gland, liver and kidney. Nevertheless, its physiological role remains uncertain because serum T3 concentrations are normal in *Dio1*$^{-/-}$ knockout mice (52). Activity of DIO2 in skeletal muscle may also contribute to circulating T3, although this role probably differs between species (6, 49, 53–55). DIO2 (Km 10^{-9} M) is more efficient than DIO1 and catalyzes outer ring deiodination to generate T3 from T4. The physiological role of DIO2 is thus to control the intracellular T3 concentration and saturation of the nuclear TR in tar-

Figure 3.



Thyroid hormone action in bone cells

(A) In hypothyroidism, despite maximum DIO2 (D2) and minimum DIO3 (D3) activities, TRα1 remains unliganded and bound to corepressor thus inhibiting T3 target gene transcription.

(B) In the euthyroid state D2 and D3 activities are regulated to optimize ideal intracellular T3 availability resulting in displacement of corepressor and physiological transcriptional activity of TRα1.

(C) In thyrotoxicosis, despite maximum D3 and minimum D2 activities, supra-physiological intracellular T3 concentrations result in increased TRα1 activation and enhanced T3 target gene responses.

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get tissues (56–58). Importantly, DIO2 protects tissues from the detrimental effects of hypothyroidism because its low Km permits efficient local conversion of T4 to T3. T4 treatment of cells in which MCT8 and DIO2 are coexpressed results in increased T3 target gene expression (59), indicating thyroid hormone uptake and metabolism coordinately regulate T3 responsiveness. By contrast, DIO3 (Km 10^{-9} M) irreversibly inactivates T3 or prevents T4 being activated by inner ring deiodination to generate T2 or rT3, respectively. The physiological role of DIO3 is thus to prevent or limit access of thyroid hormones to specific tissues at critical times during development and in tissue repair (6, 49, 51).

Consistent with this, DIO2 and DIO3 are expressed in T3-target cells, including the central nervous system (CNS), cochlea, retina, heart and skeleton (49, 60–65), and expression of both enzymes is regulated in a temporospatial and tissue-specific manner (51, 66, 67). Acting together, DIO2 and DIO3 thus control cellular T3 availability (49). For example, during fetal growth, high levels of DIO3 in placenta, uterus and fetal tissues protect developing organs from exposure to inappropriate levels of T3 and facilitate cell proliferation (68). At birth, DIO3 declines rapidly while expression of DIO2 increases to trigger cell differentiation and tissue maturation during postnatal development (49–51). The temporo-spatial and tissue-specific regulated expression of both DIO2 and DIO3 (66) and the TRα and TRβ nuclear receptors (69) combine to provide a complex and co-ordinated system for fine control of T3 availability and action in individual cell types.

F. Nuclear actions of thyroid hormones

TRα and TRβ are members of the nuclear receptor superfamily (70, 71), acting as ligand-inducible transcription factors that regulate expression of T3-target genes (Figure 3). In mammals, *THRA* encodes three C-terminal variants of TRα. TRα1 is a functional receptor that binds both DNA and T3, whereas TRα2 and TRα3 fail to bind T3 and act as antagonists in vitro (72). A promoter within intron 7 of mouse *Thra* gives rise to two truncated variants, TR$\Delta\alpha$1 and TR$\Delta\alpha$2, which are potent dominant-negative antagonists in vitro, although their physiological role is unclear (73). Two truncated TRα1 proteins p28 and p43 arise from alternate start codon usage and are proposed to mediate T3 actions in mitochondria or nongenomic responses (74, 75). *THRB* encodes two N-terminal TRβ variants, TRβ1 and TRβ2, both of which act as functional receptors. Two further transcripts, TRβ3 and TR$\Delta\beta$3, have been described but their physiological role is uncertain (76, 77). TRα1 and TRβ1 are expressed widely, but their relative concentrations differ during development and in adulthood due to tissue-specific and temporospatial regulation (69), so that most T3-target tissues are either predominantly TRα1 or TRβ1 responsive or lack isoform specificity. Expression of TRβ2, however, is markedly restricted. In the hypothalamus and pituitary, it mediates inhibitory actions of thyroid hormones on TRH and TSH expression to control the HPT axis (8, 78), while in cochlea and retina TRβ2 is an important regulator of sensory development (79, 80).

In the nucleus, TRs form heterodimers with retinoid X receptors (RXR) and bind T3 response elements (TREs) in target gene promoters to regulate transcription. Unliganded TRs compete with T3-bound TRs for DNA response elements. They are potent transcriptional repressors and have critical roles during development (81–84). Unliganded TRs interact with corepressor proteins, including nuclear receptor corepressor (NCoR) and the silencing mediator for retinoid and TR (SMRT), which recruit histone deacetylases and inhibit gene transcription (85, 86). Ligand-bound TRs interact with steroid receptor coactivator 1 (SRC1) and other related coactivators in a hormone-dependent fashion leading to target gene activation. The opposing chromatin-modifying effects of unliganded and liganded TRs greatly enhance the magnitude of the transcriptional response to T3 (87–89). In addition to positive stimulatory effects, T3 also mediates transcriptional repression to inhibit expression of key target genes, including TSH. Although negative regulatory effects are physiologically critical, underlying molecular mechanisms have not been fully characterized (88).

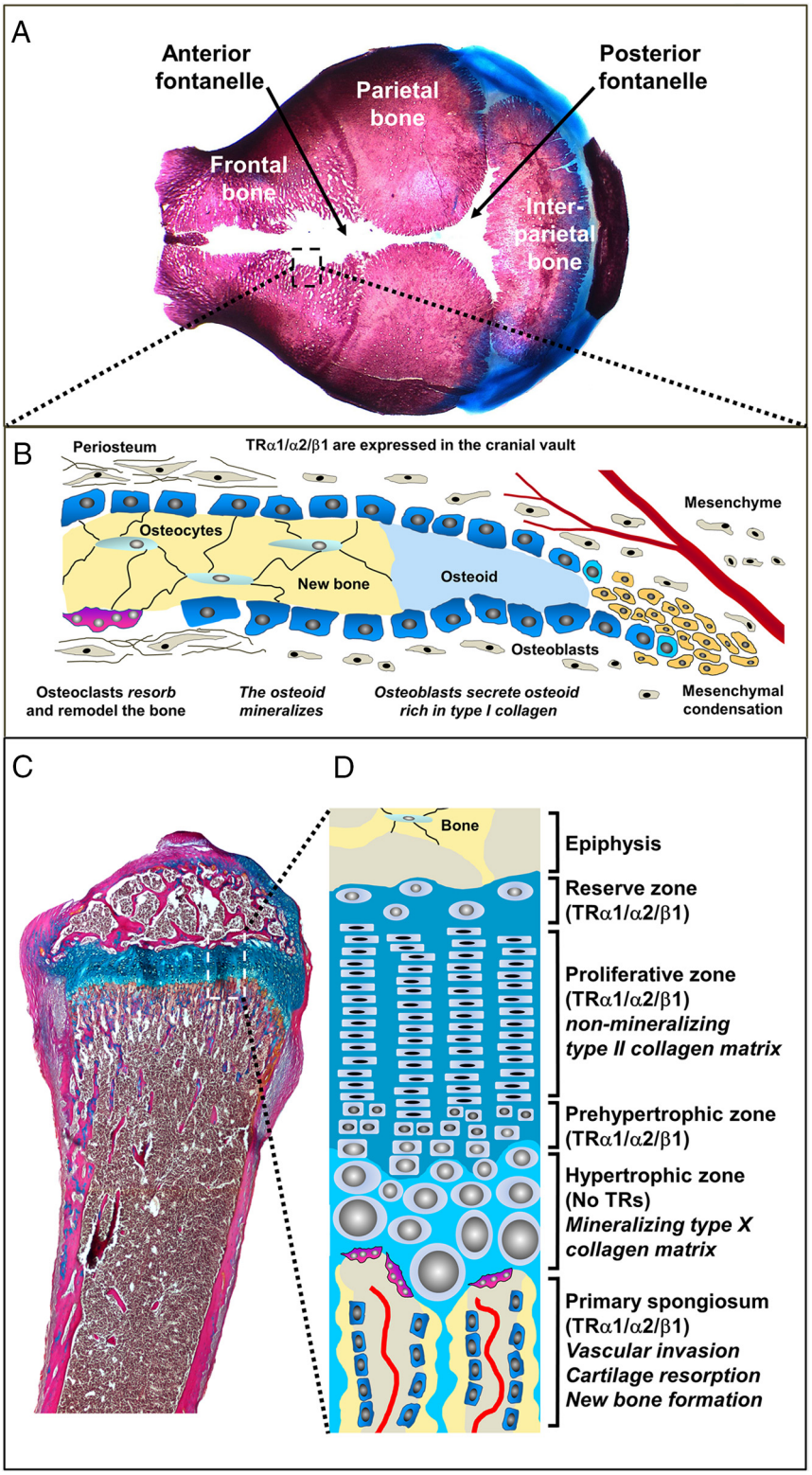
G. Nongenomic actions of thyroid hormones

Nongenomic effects of thyroid hormones include actions that do not directly influence nuclear gene expression. Nongenomic actions frequently have a short latency, are not affected by inhibitors of transcription and translation, and have agonist and antagonist affinity and kinetics divergent from classical nuclear hormone actions (90). These rapid responses are associated with second messenger pathways including (i) the phospholipase C (PLC), inositol triphosphate (IP3), diacyl glycerol (DAG), protein kinase C (PKC) and intracellular Ca^{2+} signaling pathway; (ii) the adenylyl cyclase, protein kinase A (PKA) and the cyclic AMP-response element binding protein (CREB) pathway; and (iii) the Ras, Raf1 serine/threonine kinase, mitogen activated protein kinase pathway.

Nongenomic actions of thyroid hormones have been described at the plasma membrane, in the cytoplasm and mitochondria (74, 88). The $\alpha V\beta 3$ integrin has been reported to mediate cell surface responses to T4 acting, for example, via the MAPK pathway to stimulate cell proliferation and angiogenesis (91, 92). TRβ also mediates

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Figure 4.



Intramembranous and endochondral ossification

(A) Postnatal day 1 skull vault stained with alizarin red (bone) and alcian blue (cartilage) showing sutures and fontanelles.

rapid responses to T3, acting via the PI3K/AKT/mTOR/p70^{S6K} and PI3K pathways (93–99), whereas palmitolyated TRα activates the nitric oxide/protein kinase G2/Src pathway to stimulate MAPK and PI3K/AKT downstream signaling responses that mediate rapid T3 actions in osteoblastic cells (75).

III. Skeletal physiology

Bones of the skull vault form directly from mesenchyme via intramembranous ossification whereas long bones develop on a cartilage scaffold by endochondral ossification (Figure 4). Four key cell types are involved in these developmental programs, and they are essential for linear growth in the postnatal period and maintenance of the skeleton in later life.

A. Bone and cartilage cell lineages

Chondrocytes

Chondrocytes are the first skeletal cell type to arise during development (100). In early embryogenesis mesenchyme precursors condense and define a template for the future skeleton. These cells differentiate into chondrocytes that proliferate and secrete a matrix containing aggrecan, elastin and type II collagen to form a cartilage anlage or model of the skeletal element. Cells at the center of the anlage stop proliferating and differentiate into prehypertrophic and then hypertrophic chondrocytes (101). Hypertrophic chondrocytes increase rapidly in size, synthesize a matrix rich in type X collagen and induce formation of calcified cartilage before finally undergoing apoptosis (102). Initiation of chondrogenesis requires bone morphogenic protein (BMP) signaling and the transcription factor SOX9 acting in association with SOX5 and SOX6. Indian hedgehog (IHH) stimulates chondrocyte proliferation directly

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but also ensures that sufficient chondrocyte proliferation occurs by increasing parathyroid hormone-related peptide (PTHrP) signaling, which inhibits chondrocyte hypertrophic differentiation. Canonical Wnt signaling promotes hypertrophic differentiation via inhibition of SOX9 whereas fibroblast growth factor (FGF) 18 inhibits both proliferation and differentiation of chondrocytes (102, 103).

Osteoblasts

Bone-forming osteoblasts comprise 5% of bone cells and derive from multipotent mesenchymal stem cells that can differentiate into chondrocytes, osteoblasts or adipocytes. Osteoblast maturation comprises precursor cell commitment, cell proliferation, type I collagen deposition and matrix mineralization. Following bone formation, osteoblasts may differentiate into bone lining cells or osteocytes, or undergo apoptosis (104, 105). In SOX9-expressing mesenchymal progenitors, osteoblastogenesis requires induction of two critical transcription factors RUNX2 and Osterix (105, 106). Subsequent differentiation is regulated by the IHH, PTH, Notch, canonical Wnt, BMP, insulin-like growth factor-1 (IGF-1) and FGF signaling pathways (105, 107, 108).

Osteocytes

Osteocytes comprise 90%–95% of bone cells and derive from osteoblasts that have become embedded in bone matrix. Osteocyte dendritic processes ramify though networks of canaliculi and sense fluid shear stresses, communicating via gap junctions (109, 110). Mechanical stresses and localized microdamage stimulate osteocytes to release cytokines and chemotactic signals, or induce apoptosis. In general, increased mechanical stress stimulates local osteoblastic bone formation, whereas reduced loading or microdamage results in osteoclastic bone resorption (111, 112). Osteocytes are thus mechano-sensors that control bone modeling and remodeling through their regulation of osteoclasts via the RANKL/RANK pathway and osteoblasts via modulation of Wnt signaling (113–115).

Osteoclasts

Osteoclasts comprise 1%–2% of bone cells. They are polarized multinucleated cells derived from fusion of mononuclear–myeloid precursors that resorb bone matrix and mineral. Attachment to bone is mediated by $\alpha V\beta 3$ integrin that interacts with bone matrix proteins. These interactions lead to formation of an actin ring and sealing zone with polarization of the osteoclast into ruffled border and basolateral membrane regions (116). Carbonic anhydrase II generates protons and bicarbonate within the osteoclast cytoplasm (117) and the HCO_3^- is exchanged for extracellular chloride at the basolateral membrane by a specific Cl^-/HCO_3^- channel. An osteoclast-specific pump (H^+-ATPase) transports protons across the ruffled border, while the CLCN7 channel transports chloride simultaneously. Within resorption lacuna, the acidic environment dissolves hydroxyapatite to release Ca^{2+} and HPO_4^{2-}, while a secreted cysteine protease, cathepsin K, digests organic bone matrix. The degradation products are endocytosed at the ruffled border, transported across the cytoplasm in tartrate-resistant acid phosphatase-rich vesicles and released at the basolateral membrane by exocytosis (117). Commitment of hematopoietic stem cells to the myeloid lineage is regulated by the PU.1 and microophthalmia-associated (MITF) transcription factors, which induce colony stimulating factor receptor (CSF-1R) expression. Macrophage colony stimulating factor/CSF-1R signaling stimulates expression of receptor activator of nuclear factor κB (RANK), leading to osteoclast precursor commitment. RANK ligand/RANK signaling induces the key transcription factors, nuclear factor κB (NFκB) and nuclear factor of activated T cells cytoplasmic 1 (NFATc1), leading to osteoclast differentiation and fusion (108, 118).

B. Intramembranous ossification

The flat bones of the face and skull form by intramembranous ossification, which occurs in the absence of a cartilage scaffold (Figure 4A-B). Mesenchyme progenitors, located within vascularized connective tissue membranes, condense into nodules and differentiate to bone-forming osteoblasts. The osteoblasts secrete an osteoid matrix of type I collagen and chondroitin sulfate which mineralizes to form an ossification center. The surrounding mesenchyme forms the periosteum and cells at the inner surface differentiate into lining osteoblasts. Progressive bone formation results in extension of bony spicules and fusion of adjacent ossification centers (119).

C. Endochondral ossification

Endochondral ossification is the process by which long bones form on a cartilage scaffold (Figure 4C-D) (101). Mesenchyme precursors condense and differentiate into chondrocytes, which proliferate and se-

(B) Schematic representation of intramembranous bone formation at a skull suture.

(C) Proximal tibial section at postnatal day 21 growth plate stained with alcian blue (cartilage) and van Gieson (bone matrix, red).

(D) Schematic representation of the growth plate.

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crete a matrix containing type II collagen and proteoglycans that forms a cartilage template. At the primary ossification center a coordinated program of chondrocyte proliferation, hypertrophic differentiation and apoptosis leads to mineralization of cartilage. Subsequently, vascular invasion and migration of osteoblasts enables replacement of mineralized cartilage with trabecular bone. Concurrently, peripheral mesenchyme precursors in the perichondrium differentiate into osteoblasts and form a collar of cortical bone. Secondary ossification centers form at the ends of long bones and remain separated from the primary ossification center by the epiphyseal growth plates where endochondral ossification continues.

D. Linear growth and bone maturation

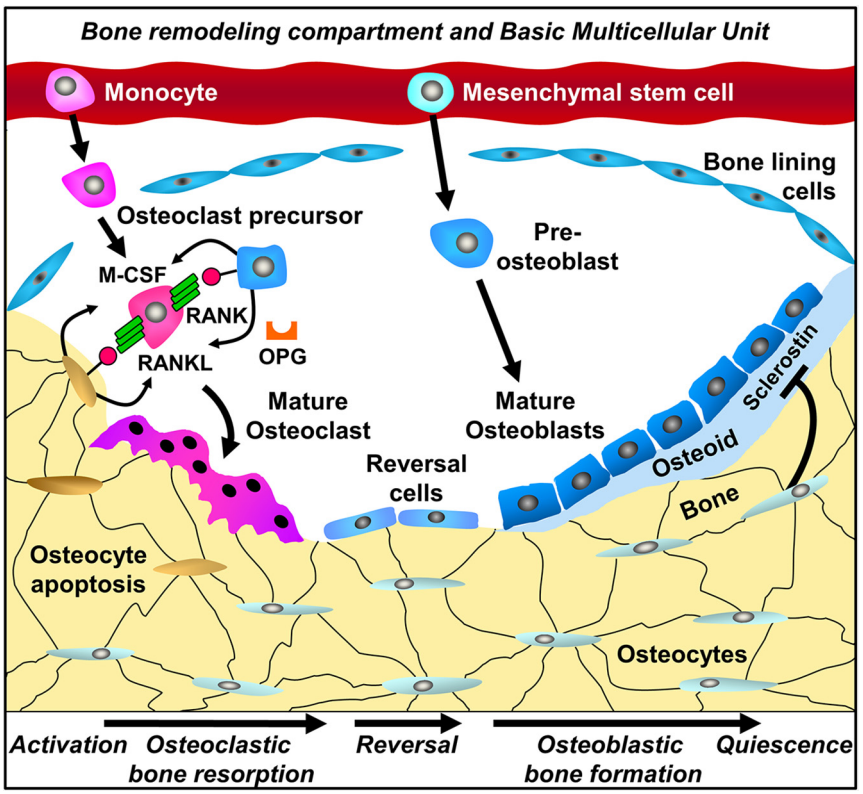
Epiphyseal growth plates at both ends of developing bones comprise the reserve, proliferative, prehypertrophic and hypertrophic zones, together with primary and secondary spongiosa (Figure 4C-D) (101). The reserve zone contains uniform chondrocytes with a low proliferation index. Cells progress to the proliferative zone, become flattened, increase type II collagen synthesis and form longitudinal columns. As chondrocytes mature they express alkaline phosphatase, undergo terminal hypertrophic differentiation, secrete type X collagen and increase in volume by 10-fold (120, 121). Finally, apoptosis of hypertrophic chondrocytes results in release of angiogenic factors that stimulate vascular invasion and migration of osteoblasts and osteoclasts, leading to remodeling of calcified cartilage and formation of trabecular bone. This ordered process mediates linear growth until adulthood (101). Synchronously, the diameter of the long bone diaphysis increases by osteoblastic deposition of cortical bone beneath the periosteum, and the marrow cavity expands as a consequence of osteoclastic bone resorption at the endosteal surface.

Progression of endochondral ossification and linear growth is tightly regulated by a local feedback loop involving IHH and PTHrP (101, 122), and other factors including systemic hormones (thyroid hormones, growth hormone (GH), IGF-1, glucocorticoids, sex steroids), various cytokines and growth factors (BMPs, FGFs, vascular endothelial growth factors) that act in a paracrine and autocrine manner (101). Linear growth continues until fusion of the growth plates during puberty, but bone mineralization and consolidation of bone mass continues until peak bone mass is achieved during the third to fourth decade (101, 123, 124).

E. The bone remodeling cycle

Functional integrity and strength of the adult skeleton is maintained in a continuous process of repair by the 'bone remodeling cycle' (125) (Figure 5). The basic multicellular unit (BMU) of bone remodeling comprises osteoclasts and osteoblasts whose activities are orchestrated by osteocytes (113, 126, 127). Over 95% of the surface of the adult skeleton is normally quiescent because osteocytes exert resting inhibition of

Figure 5.



Bone remodeling compartment and "Basic Multicellular Unit" of the bone remodeling cycle.

The bone remodeling cycle is initiated and orchestrated by osteocytes. Bone remodeling results from changes in mechanical load, structural microdamage or exposure to systemic or paracrine factors. Monocyte/macrophage precursors differentiate to mature osteoclasts and resorb bone. Differentiation is induced by macrophage colony-stimulating factor (CSF) (M-CSF) and receptor activator of NFkB ligand (RANKL) and inhibited by osteoprotegerin (OPG). During reversal, osteoblastic progenitors are recruited to the site of resorption, synthesize osteoid, and mineralize new bone to repair the defect.

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both osteoclastic bone resorption and osteoblastic bone formation (117).

Under basal conditions, osteocytes secrete transforming growth factor-β (TGFβ) and sclerostin, which inhibit osteoclastogenesis and Wnt-activated osteoblastic bone formation, respectively. Increased load or local microdamage results in a fall in local TGFβ levels (128) and activation of bone lining cells leads to recruitment of osteoclast progenitors. Osteocytes and bone lining cells express M-CSF and RANKL, the two cytokines required for osteoclastogenesis (114, 125). RANKL acts via several downstream signaling molecules, including c-fos, NF-kB, NFATc1, MAPK and TNF receptor-associated factor-6 (129–131). RANKL also induces expression of αVβ3 integrin in osteoclast precursors, which signals via c-src to induce activation of small GTPases that are critical for formation of the actin ring sealing zone and osteoclast migration and survival. In addition to RANKL, osteoblasts and bone marrow stromal cells express osteoprotegerin (OPG). OPG is a secreted decoy receptor for RANKL and functions as the physiological inhibitor of RANK–RANKL signaling (117, 132). Thus, the RANKL: OPG ratio determines osteoclast differentiation and activity. This ratio is regulated by systemic hormones and local cytokines that control bone remodeling and include estrogen, PTH, glucocorticoids, TNF-α, IL-1 and prostaglandin E2 (133).

Following this 30–40 day resorption phase, reversal cells remove undigested matrix fragments from the bone surface, and local paracrine signals released from degraded matrix recruit osteoblasts that initiate bone formation. Over the next 150 days, osteoblasts secrete and mineralize new bone matrix (osteoid) to fill the resorption cavity. Although commitment of mesenchyme precursors to the osteoblast lineage requires both Wnt and BMP signaling, the canonical Wnt pathway subsequently acts as the master regulator of osteogenesis (134–136). Physiological negative regulation of canonical Wnt signaling is mediated by the osteocyte, which secretes soluble factors (sclerostin, Dickkopf1-related protein 1 (DKK1) and secreted frizzled related protein 1 (SFRP1)) that interfere with the interaction between Wnt ligands and their receptor and coreceptor (113, 126). During the process of bone formation, some osteoblasts become embedded within newly formed bone and undergo terminal differentiation to osteocytes. Secretion of sclerostin and other Wnt inhibitors by these osteocytes leads to cessation of bone formation and a return to the quiescent state in which osteoblasts become bone-lining cells (113, 126).

This cycle of targeted bone modeling and remodeling enables the adult skeleton to repair old or damaged bone, react to changes in mechanical stress and respond rapidly to the demands of mineral homeostasis.

IV. Skeletal target cells and downstream signaling pathways

A. TSH actions in chondrocytes, osteoblasts and osteoclasts

Expression of TSHR and its ligands in skeletal cells

TSHR is expressed predominantly in thyroid follicular cells, but expression in chondrocytes, osteoblasts and osteoclasts suggests TSH exerts direct actions in cartilage and bone (32, 137). Although pituitary TSH functions as a systemic hormone, local expression of TSHR ligands in bone has also been investigated. TSHα and TSHβ subunits are not expressed in primary human or mouse osteoblasts or osteoclasts (138, 139). Nevertheless, an alternative splice variant of *Tshb* (Tshb-sv) has been identified in mouse bone marrow (140, 141). Expression of this variant in bone marrow-derived macrophages activated cAMP in cocultured, stably transfected TSHR-overexpressing CHO cells (142). mRNA encoding the isoform was also identified at low levels in primary mouse osteoblasts but not osteoclasts (139). The alternative TSHR ligand, thyrostimulin, is also expressed in osteoblasts and osteoclasts, and studies of *Gpb5*$^{-/-}$ mice lacking thyrostimulin indicated thyrostimulin regulates osteoblastic bone formation during early skeletal development. However, the underlying mechanisms remain unknown, as thyrostimulin failed to influence osteoblast proliferation or differentiation, or activate cAMP, ERK, P38 MAPK or AKT signaling pathways in primary osteoblasts or bone marrow stromal cells in vitro (139).

Chondrocytes

Only limited information has been published regarding the TSHR in cartilage. In mesenchymal stem cells, TSH stimulated self-renewal and expression of chondrogenic marker genes suggesting TSH may increase chondrocyte differentiation (143). Growth plate cartilage and cultured chondrocytes express TSHR, and treatment with TSH increased cAMP activity and decreased expression of SOX9 and type IIa collagen expression in primary chondrocytes (137).

Initial studies, therefore, suggest TSHR signaling might inhibit chondrocyte differentiation (Figure 6).

Osteoblasts

Expression of TSHR in UMR106 rat osteosarcoma cells was reported in 1998 (144) and, subsequently, expression of *TSHR* mRNA and protein was identified in osteoblasts and osteoclasts (32, 38, 138, 139, 145). The

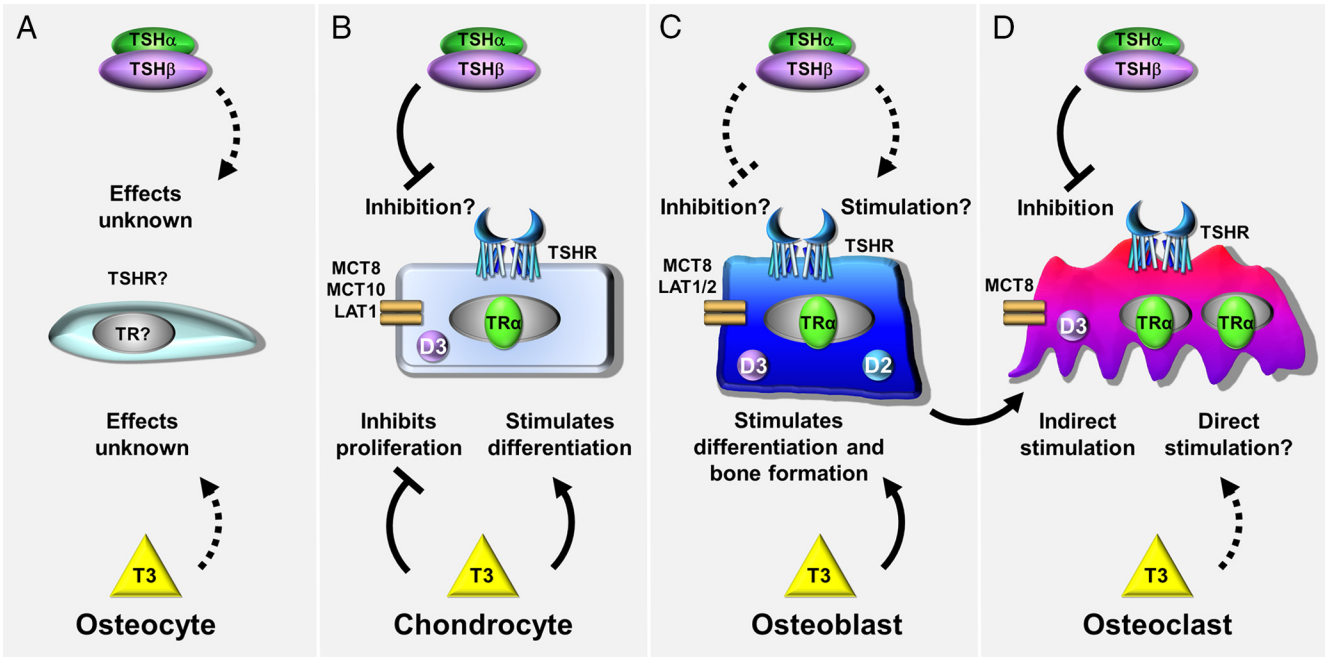
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lack of TSHα and β expression in osteoblasts and osteoclasts (138, 139), indicates TSH does not have local autocrine effects in these cells. Nevertheless, treatment of osteoblasts with TSH in vitro inhibited osteoblastogenesis and reduced expression of type I collagen, bone sialoprotein and osteocalcin (32). Inhibition of low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) mRNA in these studies suggested the effects of TSH on osteoblastogenesis and function might be mediated via Wnt signaling (32).

By contrast, Sampath et al and Baliram et al showed that TSH stimulates osteoblast differentiation and function (142, 145). Furthermore, in ES cell cultures, TSH stimulated osteoblastic differentiation via protein kinase C and the noncanonical Wnt pathway components Frizzled and Wnt5a (146). In human SaOS2 osteosarcoma cells, TSH also stimulated proliferation and differentiation as measured by alkaline phosphatase, and increased IGF-1 and IGF-II mRNA expression together with complex regulatory effects on IGFBPs and their proteases (147). Finally, TSH stimulated β-arrestin 1 leading to activation of ERK, P38 MAPK and AKT signaling pathways and osteoblast differentiation in stably transfected human osteoblastic U2OS-TSHR cells that overexpress the TSHR (148).

Despite these contrasting findings, Tsai et al had previously shown only low levels of TSHR expression, TSH binding and cAMP activation in human osteoblasts and concluded TSH was unlikely to have a physiological role (149). Further studies also demonstrated only low levels of TSHR protein in calvarial osteoblasts, and in these studies treatment with TSH and TSHR-stimulating antibodies failed to induce cAMP and TSH did not affect osteoblast differentiation or function (38, 138).

Figure 6.



Actions of T3 and TSH in skeletal cell

(A) T3 and TSH actions in osteocytes have not been investigated and it is unknown whether osteocytes express thyroid hormone transporters, deiodinases, TRs or the TSHR.

(B) Chondrocytes express MCT8, MCT10 and LAT1 transporters, DIO3 (D3), TRs (predominantly TRα), and TSHR. T3 inhibits proliferation and stimulates prehypertrophic and hypertrophic chondrocyte differentiation, while TSH might inhibit proliferation and matrix synthesis.

(C) Osteoblasts express MCT8 and LAT1/2 transporters, the DIO2 (D2) and D3, TRs (predominantly TRα), and TSHR. Most studies indicate T3 stimulates osteoblast differentiation and bone formation. Contradictory data suggest TSH may stimulate, inhibit or have no effect on osteoblast differentiation and function.

(D) Osteoclasts express MCT8, D3, TRs and the TSHR. Currently it is unclear if T3 acts directly in osteoclasts or whether indirect effects in the osteoblast lineage mediate its actions. Most studies indicate TSH inhibits osteoclast differentiation and function.

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Overall, findings have been interpreted to suggest that changes in TNFα, RANKL, OPG and interleukin 1 signaling in response to TSH might be mediated via an alternative G-protein and not by cAMP (32, 38, 150). Thus, although the TSHR is expressed in osteoblasts, in vitro data are contradictory suggesting TSH may inhibit, enhance or have no effect on osteoblast differentiation and function (Figure 6). Furthermore, the physiological second messenger pathway that lies downstream of activated TSHR in osteoblasts has not yet been defined.

Osteoclasts

Bassett et al showed mouse osteoclasts express low levels of TSHR protein, but treatment with TSH and TSHR-stimulating antibodies did not affect osteoclast differentiation and function or elicit a cAMP response (138). Nevertheless, most studies have shown TSH inhibits osteoclast formation and function. Thus, treatment of monocyte precursors with TSH inhibited osteoclastogenesis and dentine resorption (32, 38, 145), while TSH and TSHR-stimulating antibodies also inhibited osteoclastogenesis in mouse embryonic stem cell (ESC) cultures treated with M-CSF, RANKL, vitamin D and dexamethasone (150). Zhang et al also showed TSH inhibits tartrate-resistant acid phosphatase (TRAP), MMP-9 and cathepsin K expression and osteoclastogenesis in RAW264.7 cells (151).

In vitro studies using bone marrow cultures from *Tshr*$^{-/-}$ mice revealed that inhibitory effects of TSH on osteoclastogenesis were mediated by TNFα acting via disruption of activator protein 1 (AP-1) and NF-κB signaling (32, 38, 152), and the pathogenesis of bone loss in *Tshr*$^{-/-}$ mice was proposed to be mediated by elevated levels of TNFα (153). Nevertheless, the mechanisms of bone loss in *Tshr*$^{-/-}$ mice appear complex and the underlying signaling pathways remain incompletely defined. Thus, TSH inhibited osteoclastogenesis in WT mice and TNFα stimulated osteoclastogenesis in WT and *Tshr*$^{-/-}$ mice. Accordingly, TSH inhibited TNFα via AP-1 and RANKL-NFκB signaling pathways in osteoclasts in vitro (153), although in previous studies TSH had been shown to stimulate TNFα in ES-cell derived osteoblasts (146). Together, these findings were proposed as a counter-regulatory mechanism of TNFα inhibition and stimulation in osteoclasts and osteoblasts, respectively (153).

Overall, most studies indicate that TSHR signaling inhibits osteoclastogenesis and function by complex mechanisms primarily involving TNFα (Figure 6).

B. T3 actions in chondrocytes, osteoblasts and osteoclasts

Expression of thyroid hormone transporters

The thyroid hormone transporter MCT8 is expressed and regulated by thyroid status in growth plate chondrocytes, osteoblasts and osteoclasts (61, 154). Recent studies indicate OATP1c1 is not expressed in the skeleton (154). MCT10 appears to be the major transporter expressed in the growth plate (155), while expression of the less specific LAT1 and LAT2 transporters has also been detected in bone (61, 154, 155). Nevertheless, the physiological importance and possible redundancy of thyroid hormone transporters in the skeleton has yet to be determined (Figure 6).

Expression of deiodinases

Thyroid hormone metabolism occurs in skeletal cells (156). Although DIO1 is not expressed in cartilage or bone (61, 156), the activating enzyme DIO2 is expressed in osteoblasts (60, 61). *Dio2* mRNA has also been detected in the embryonic mouse skeleton as early as embryonic day E14.5 and increases until E18.5 (157, 158). In the developing chick growth plate, DIO2 activity is restricted to the perichondrium (159), indicating the enzyme has a role in local regulation of thyroid hormone signaling during fetal bone development. DIO2 is also expressed in primary mesenchymal stem cells, in which expression is strongly induced following treatment with BMP-7 (160). The inactivating DIO3 enzyme is present in all skeletal cell lineages particularly during development, with the highest levels of activity in growth plate chondrocytes prior to weaning (61, 157). Together, these data suggest that control of tissue T3 availability by DIO2 and DIO3 is likely to be important for skeletal development, linear growth and osteoblast function (Figure 6).

Expression of thyroid hormone receptors

TRs are expressed at sites of intramembranous and endochondral bone formation. The localization of TR proteins to reserve and proliferative zone growth plate chondrocytes, but not hypertrophic cells (161, 162), suggests that progenitor cells and proliferating chondrocytes are primary T3-target cells but differentiated chondrocytes lose the ability to respond to T3. Both TRα1 and TRβ1 are expressed in bone and quantitative RT-PCR studies reveal that levels of TRα1 are at least 10-fold greater than TRβ1 (163, 164), suggesting TRα1 is the predominant mediator of T3 action in bone. Nevertheless, other studies also indicate TRβ may play a role (165–167).

TRα1, TRα2, and TRβ1 are expressed in reserve and proliferative zone epiphyseal growth plate chondrocytes (161, 162, 168–173) and in immortalized osteoblastic cells from several species (170, 174–181), as well as in primary osteoblasts and osteoblastic bone marrow stromal cells (175, 182, 183). However, it is unknown whether TRs are expressed in osteocytes (184, 185). Thyroid hormones stimulate osteoclastic bone resorption

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(186, 187), but this effect may be indirect and mediated by T3-responsive osteoblasts (188, 189). Although immunolocalization of TR proteins and detection of TR mRNAs by in situ hybridization in osteoclasts from pathological human osteophytes and osteoclastoma tissue were reported in early studies (168, 174, 190), TR antibodies lack sufficient sensitivity to detect expression of endogenous protein and it remains uncertain whether osteoclasts express functional TRs or respond directly to T3.

Overall, current studies indicate that reserve zone and proliferating chondrocytes, osteoblastic bone marrow stromal cells and osteoblasts are major T3-target cells in bone and predominantly express TRα (Figure 6).

Chondrocytes

Hypertrophic chondrocyte differentiation and vascular invasion of cartilage are sensitive to thyroid status (191); findings that support early studies (192–194) and reinforce the critical importance of T3 for endochondral ossification and linear growth. Nevertheless, studies of T3 action in chondrocytes cultured in monolayers are conflicting due to the species, source of chondrocytes, and culture conditions studied (171, 195–199). Consequently, several three-dimensional culture systems have been devised to investigate the T3-regulated differentiation potential of chondrocytes in vitro (196, 200–202). T3 treatment of chondrogenic ATDC5 cells, mesenchymal stem cells, primary growth plate chondrocytes and long bone organ cultures inhibits cell proliferation and concomitantly stimulates hypertrophic chondrocyte differentiation and cellular apoptosis (161, 197, 203–209). T3 promotes hypertrophic differentiation by induction of cyclin-dependent kinase inhibitors to regulate the G1-S cell cycle checkpoint (200). Subsequently, T3 stimulates BMP4 signaling, synthesis of a collagen X matrix, and expression of alkaline phosphatase and MMP13 to facilitate progression of hypertrophic differentiation and cartilage mineralization (120, 161, 197, 203–206). In addition, T3 regulation of growth plate chondrocyte proliferation and differentiation in vitro involves activation of IGF-1 and Wnt signaling (210–212).

The regulatory effects of T3 on endochondral ossification and linear growth in vivo involve interactions with key pathways that regulate growth plate maturation including IHH, PTHrP, IGF1, Wnt, BMPs, FGFs and leptin (213–215). IHH, PTHrP and BMP receptor-1A participate in a negative feedback loop that promotes growth plate chondrocyte proliferation and inhibits differentiation thereby controlling the rate of linear growth. The set-point of this feedback loop is sensitive to thyroid status (162, 216) and regulated by local thyroid hormone metabolism and T3 availability (159). Furthermore, T3 stimulates expression of genes involved in cartilage matrix synthesis, mineralization and degradation; including matrix proteoglycans (203, 204, 217–221) and collagen degrading enzymes such as aggrecanase-2 (a disintegrin and metalloproteinase with thrombospondin motifs1, ADAMTS5) and MMP13 (205, 222, 223), as well as BMP4, Wnt4 and FGFR3 (120, 210, 224–226).

In summary, thyroid hormone is essential for coordinated progression of endochondral ossification, acting to stimulate genes that control chondrocyte maturation and cartilage matrix synthesis, mineralization and degradation.

Osteoblasts

Although primary osteoblasts (170, 172, 227–233) and several osteoblastic cell lines (176, 178–181, 223, 234–245) respond to T3 in vitro, the consequences of T3 stimulation vary considerably and depend on species, the anatomical origin of osteoblasts (183, 246–248), cell type, passage number, cell confluence, stage of differentiation, and the dose and duration of T3 treatment. Thus, T3 has been shown to stimulate, inhibit, or have no effect on osteoblastic cell proliferation. A general consensus, however, indicates that T3 stimulates osteoblast proliferation and differentiation and bone matrix synthesis, modification and mineralization. T3 increases expression of osteocalcin, osteopontin, type I collagen, alkaline phosphatase, IGF-I and its regulatory binding proteins (IGF1BP-2 and –4), interleukin-6 and –8, MMP9, MMP13, tissue inhibitor of metalloproteinase- 1 (TIMP-1), FGFR1 leading to activation of MAPK-signaling, and also regulates the Wnt pathway (165, 179, 180, 223, 227, 229, 232, 235–237, 243–245, 249–259). Furthermore, IGFBP-6 interacts directly with TRα1 to inhibit T3-stimulated increases in alkaline phosphatase activity and osteocalcin mRNA in osteoblastic cells (260). Thus, T3 stimulates osteoblast activity both directly and indirectly via complex pathways involving many growth factors and cytokines. T3 may also potentiate osteoblast responses to PTH (233) by modulating expression of PTH/PTHrP receptor (176).

Despite the many potential T3-target genes identified in osteoblasts, little information is available regarding mechanisms by which their expression is modulated, and T3 regulation may involve other signaling pathways. For example, T3 regulates osteoblastic cell morphology, cytoskeleton, and cell-cell contacts in vitro (234, 239, 240). In addition, T3 stimulates osteocalcin via nongenomic actions mediated by suppression of Src (261). T3 also phosphorylates and activates p38 MAPK and stimulates osteocalcin expression in MC3T3 cells (250, 262), a pathway that is enhanced by AMPK activation (263) but inhibited by cAMP (264) and Rho-kinase (265). A recent

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study further demonstrated that nongenomic signaling in osteoblasts and osteosarcoma cells is mediated by a plasma membrane bound N-terminal truncated isoform of TRα1 that is palmitoylated and interacts with caveolin-containing membrane domains. Acting via this isoform, T3 stimulated osteoblast proliferation and survival via increased intracellular Ca^{2+}, NO and cGMP leading to activation of protein kinase GII, Src and ERK (75).

Overall, many studies in primary cultured and immortalized osteoblastic cells demonstrate the complexities of T3 action in bone and emphasize the importance of the cellular system under study. Many of these T3 actions involve interactions with bone matrix and local paracrine and autocrine factors via mechanisms that have yet to be determined.

Osteoclasts

Thyroid hormone excess results in increased osteoclast numbers and activity in vivo leading to bone loss. Osteoclasts express TRα1 and TRβ1 mRNAs but it is not clear whether functional receptors are expressed because TR antibodies lack sufficient sensitivity to detect endogenous proteins. Studies of mixed cultures containing osteoclast lineage cells and bone marrow stromal cells have been contradictory and it is not clear whether stimulation of osteoclastic bone resorption results from direct T3-actions in osteoclasts or indirect effects mediated by primary actions in cells of the osteoblast lineage (186–188, 254, 266). Studies of fetal long bone and calvarial cultures (173, 267, 268) implicated various cytokines and growth factors including IGF-1 (269, 270), prostaglandins (186), interleukins (271), TGFβ (238, 272), and interferon-γ (186) as mediators of secondary responses in osteoclasts. Similarly, treatment of immortalized osteoblasts or primary bone marrow stromal cells resulted in increased RANKL, interleukin 6 (IL-6), IL-8 and prostaglandin E2 expression, and inhibition of OPG, consistent with an indirect effect of thyroid hormones on osteoclast function (254, 258, 266). Other studies, however, suggest effects of T3 on osteoclastogenesis are independent of RANKL signaling (273, 274). A further complication is that while TR expression is well documented in osteoblastic cells, some of the effects of T3 on bone organ cultures are extremely rapid and involve mobilization of intracellular calcium stores to suggest that nongenomic TR-independent actions of T3 may be relevant (275).

Overall, it is unclear whether T3 acts directly in the osteoclast lineage, or whether its stimulatory effects on osteoclastogenesis and bone resorption are secondary responses to direct actions of T3 in osteoblasts, osteocytes, stromal cells or other bone marrow cell lineages.

V. Genetically modified mice (Table 1)

A. Targeting TSHR signaling

Skeletal development and growth

TSHR knockout (*Tshr*$^{-/-}$) mice have congenital hypothyroidism with undetectable thyroid hormones and 500-fold elevation of TSH. *Tshr*$^{-/-}$ mice are growth retarded and usually die by 4 weeks of age (276). Nevertheless, animals supplemented with thyroid extract from weaning regain normal weight by 7 weeks. Heterozygous *Tshr*$^{+/-}$ mice are euthyroid with normal linear growth. Untreated *Tshr*$^{-/-}$ mice had a 30% reduction in BMD with evidence of increased bone formation and resorption when analyzed during growth at 6 weeks of age. *Tshr*$^{-/-}$ mice treated with thyroid extract displayed a 20% reduction in BMD and reduced calvarial thickness, although histomorphometry responses were not reported (32). Heterozygotes had a 6% reduction in total BMD, affecting only some skeletal elements, no change in calvarial thickness and no difference in parameters of bone resorption or formation. These studies were interpreted to indicate that TSH suppresses bone remodeling, and TSH was proposed as an inhibitor of bone formation and resorption (32). Normally, T4 and T3 levels rise rapidly to a physiological peak at 2 weeks of age in mice, and growth velocity is maximal at this time (277, 278). Since *Tshr*$^{-/-}$ mice are only supplemented with thyroid extract from weaning at around 3 weeks of age (32, 276), they remain grossly hypothyroid at this critical stage of skeletal development. Thus, the phenotype reported in *Tshr*$^{-/-}$ mice also reflects the effects of severe hypothyroidism followed by incomplete "catch-up" growth and accelerated bone maturation in response to delayed thyroid hormone replacement (138, 279–281). Furthermore, treatment with supraphysiological doses of T4 for 21 days resulted in increased bone resorption and a greater loss of bone in *Tshr*$^{-/-}$ mice compared to wild-type controls, suggesting *Tshr* deficiency exacerbates bone loss in thyrotoxicosis (140).

To investigate the relative importance of T3 and TSH in bone development, two contrasting mouse models of congenital hypothyroidism were compared, in which the reciprocal relationship between thyroid hormones and TSH was either intact or disrupted (138). *Pax8*$^{-/-}$ mice lack a transcription factor required for thyroid follicular cell development (282) and hyt/hyt mice harbor a loss-of-function mutation in *Tshr* (283). *Pax8*$^{-/-}$ mice have a 2000-fold elevation of TSH (277, 284) and a normal TSHR, whereas hyt/hyt mice have a 2000-fold elevation of TSH but a nonfunctional TSHR. Thus, if TSHR has the predominant role these mice should display opposing skeletal phenotypes. However, *Pax8*$^{-/-}$ and hyt/hyt mice each displayed

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Table 1. Skeletal phenotype of genetically modified mice

Mouse model	Genotype	Systemic thyroid status	Developing skeleton	Adult skeleton
Congenital Hypothyroid				
Pax8$^{-/-}$	Maximal Tshr signaling Apo TRα and TRβ	*No Thyroid*	Impaired linear growth, delayed endochondral ossification, reduced cortical bone, reduced bone mineralization	Majority die by weaning;
		T_4 undetectable		
		T_3 undetectable		
		TSH 1900x		
TSHR mutants				
Tshr$^{-/-}$	No Tshr	*Thyroid hypoplasia*	Severe growth retardation; impaired linear growth; reduced mineral density; increased bone resorption; increased bone formation or decreased bone formation	Die by weaning unless tr
	Apo TRα and TRβ	T_4 undetectable		Low bone mass, high bo
		T_3 undetectable		Enhanced bone loss in su
		TSH >500x		
Tshr$^{P556L/P556L}$	Absent Tshr signaling	*Thyroid hypoplasia*	Impaired linear growth, delayed endochondral ossification, reduced cortical bone, reduced bone mineralization	*NR*
(hyt/hyt)	Apo TRα and TRβ	T_4 0.06x		
		T_3 0.06x		
		TSH 2300x		
Gpb5$^{-/-}$	No Thyrostimulin	*Juveniles*	Normal linear growth; endochondral and intramembranous ossification. Increased bone volume and mineralization due to increased osteoblastic bone formation	Skeletal phenotype resol by early adulthood
	(Alternative high affinity Tshr ligand)	T_4 0.7x, (males only)		
		T_3 normal		
		TSH 3 × (males only)		
		Adults		
		T_4,T_3 and TSH normal		
Compound mutants				
Tshr$^{-/-}$Tnf$\alpha^{-/-}$	No Tshr or TNFα	*Treated with thyroid extract from weaning*	*NR*	Amelioration of low bon high bone turnover phen
		T_4,T_3 and TSH not reported		
TR mutants				
TRα mutants				
TR$\alpha^{-/-}$	No TRα1 or TRα2 TR$\Delta\alpha$1and TR$\Delta\alpha$2 preserved	*Hypothyroid*	Severe growth delay; delayed endochondral ossification; impaired chondrocyte differentiation; reduced mineralization	Die by weaning unlessT_3
		T_4 0.1x		
		T_3 0.4x		
		TSH 2x		
		(GH normal)		
TRα1$^{-/-}$	No TRα1 or TR$\Delta\alpha$1 TRα2 and TR$\Delta\alpha$2 preserved	*Mild hypothyroidism*	No growth retardation	*NR*
		T_4 0.7 × (males only)		
		T_3 normal,		
		TSH 0.8x		
TRα2$^{-/-}$	No TRα2 or TR$\Delta\alpha$2	*Mild hypothyroidism*	No growth retardation	Reduced bone mineral d
	TRα1 and TR$\Delta\alpha$1 over-expression	T_4 0.8x	normal endochondral ossification	
		T_3 0.7x		
		TSH normal		
		(GH normal IGF1 low)		
TRα1$^{GFP/GFP}$	No TRα2	*Euthyroid*	Normal post natal growth	*NR*
	2 × increase in TRα1^{GFP}	T_4, T_3 and TSH normal		
TR$\alpha^{0/0}$	No TRα	*Euthyroid*	Transient growth delay, delayed endochondral ossification, impaired chondrocyte differentiation, reduced mineral deposition	Osteosclerosis, increased bone volume, reduced os bone resorption
	Normal TRβ	T_4 normal		
		T_3 normal		
		TSH normal		
		(GH normal)		
TRα1$^{PV/+}$	Heterozygous dominant-negative TRα receptor	*Euthyroid*	Severe persistent growth retardation; delayed intramembranous and endochondral ossification; impaired chondrocyte differentiation; reduced mineralization	Grossly dysmorphic bone cortical bone volume; red
		T_4 normal		*(Resistant to T_4 treatmer*
		T_3 1.2x		
		TSH 1.5–2x		
		(GH normal)		
TRα1$^{R384C/+}$	Heterozygous dominant-negative TRα receptor	*Euthyroid adults*	Transient growth delay; delayed intramembranous and endochondral ossification; impaired chondrocyte differentiation	Impaired bone modelling
	(10 × lower affinity for T_3)	*Mild hypothyroidism (P10–35)*		*(Ameliorated T_3 treatme*
		T_4 0.8x		
		T_3 0.7x		
		TSH 0.7x		
		(GH reduced in juveniles)		
TRα1$^{R398H/+}$	Heterozygous dominant-negative TRα receptor	*Euthyroid juveniles*	*NR*	*NR*
		T_4 and T_3 normal		
		TSH 3.4x		
TRα1$^{L400R/+}$	Global expression	*Euthyroid*	Severe persistent growth retardation; delayed endochondral ossification	*NR*
($TR^{AMI/+}$xSycp1-Cre)	dominant-negative	T_4 and T_3 normal		
	receptor TRα1^{L400R}	TSH normal		
		(GH low)		
TRα1$^{L400R/+}$/C1	Osteoblast expression dominant-negative	*Euthyroid*	No skeletal abnormalities reported following limited analysis	No skeletal abnormalities
($TR^{AMI/+}$x Col1a1-Cre)	receptor TRα1^{L400R}	T_4 and T_3 and TSH normal		
TRα1$^{L400R/+}$/C2	Chondrocyte and osteoblast expression dominant-negative	*Euthyroid*	Persistent growth retardation	Short stature
($TR^{AMI/+}$x Col2a1-Cre)	receptor TRα1^{L400R}	T_4 and T_3 and TSH normal	Delay in endochondral ossification	
			Skull abnormalities	
			Reduced cortical and trabecular bone	
			Decreased mineralization	
TRβ mutants				

(*Continued*)

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Table 1. Continued

Mouse model	Genotype	Systemic thyroid status	Developing skeleton	Adult skeleton
$TR\beta^{-/-}$	No TRβ	*RTH and goitre*	Persistent short stature, advanced endochondral and intramembranous ossification, increased mineral deposition	Osteoporosis, redu
	Normal TRα	T_4 3–4x T_3 3–4x TSH 2.6–8x		
$TR\beta2^{-/-}$	No TRβ2 TRβ1 preserved	*Mild RTH* T_4 1–3x T_3 1.3–1.5x TSH 1.2–2.5x	No growth abnormality	*NR*
$TR\beta^{PV/PV}$	Homozygous dominant-negative TRβ receptor	*Severe RTH and goitre*	Accelerated prenatal growth; persistent postnatal growth retardation; advanced intramembranous and endochondral ossification; increased mineralization	*NR*
		T_4 15x T_3 9x TSH 400x		
$TR\beta^{\Delta 337T/\Delta 337T}$	Homozygous dominant-negative TRβ receptor	*Severe RTH and goitre* T_4 15x T_3 10x TSH 50x	No growth phenotype	*NR*
TRβ transgenics				
*Tshb***[GRAPHIC]**$TR\beta^{G345R}$	Pituitary expression of dominant-negative $TR\beta^{G345R}$	*RTH* T_4 1.2x TSH normal	Normal growth	*NR*
Cga**[GRAPHIC]**$TR\beta^{\Delta 337T}$	Pituitary expression of dominant-negative $TR\beta^{\Delta 337T}$	T_4 normal TSH 3x *(Reduced bioavailability?)*	*NR*	*NR*
Actb**[GRAPHIC]**$TR\beta^{PV}$	ubiquitous expression of dominant-negative $TR\beta^{PV}$	*RTH* T_4 1.5x TSH normal	Impaired weight gain	*NR*
Cga**[GRAPHIC]**$TR\beta^{PV}$	Pituitary expression of dominant-negative $TR\beta^{PV}$	*Euthyroid* T_4, T_3 and TSH normal	Impaired weight gain	*NR*
Compound mutants				
$TR\alpha^{-/-}TR\beta^{-/-}$	No TRα1, TRα2 or TRβ	*RTH and small goitre*	Growth delay similar to $TR\alpha^{-/-}$; delayed endochondral ossification; impaired chondrocyte differentiation;	Die at or near wea
	TRΔα1 and TRΔα2 preserved	T_4 10x T_3 10x TSH >100x	reduced mineralization	
$TR\alpha1^{-/-}TR\beta^{-/-}$	No TRα1, TRΔα1 or TRβ	*RTH and large goitre*	Persistent growth retardation; delayed endochondral ossification; reduced mineralization	Reduced trabecula
	TRα2 and TRΔα2 preserved	T_4 60x T_3 60x TSH >160x *(GH/IGF1 low)*		mineral density GH treatment corr
$TR\alpha2^{-/-}TR\beta^{-/-}$	No TRα2, TRΔα2 or TRβ TRα1 and TRΔα1 over-expression	*Mild hypothyroidism* T_4 0.7x T_3 0.8x TSH normal	Transient growth delay	*NR*
$TR\alpha^{0/0}TR\beta^{-/-}$	No TRα or TRβ	*RTH and goitre* T_4 14x T_3 13x TSH >200x; *(GH/IGF1 low)*	More severe phenotype than $TR\alpha^{0/0}$; growth delay; delayed endochondral ossification; impaired chondrocyte differentiation; reduced mineralization	*NR*
$Pax8^{-/-}TR\alpha1^{-/-}$	No TRα1 or TRΔα1 TRα2/TRΔα2 preserved Apo TRβ Maximal Tshr signaling	*No Thyroid* T_4 undetectable T_3 undetectable TSH *NR*	Growth retardation similar to $Pax8^{-/-}$	Die by weaning
$Pax8^{-/-}TR\alpha^{0/0}$	No TRα Apo TRβ Maximal Tshr signaling	*No Thyroid* T_4 undetectable T_3 undetectable TSH >400x	Growth retardation less than $Pax8^{-/-}$ and similar to $TR\alpha^{0/0}\beta^{-/-}$; delayed endochondral ossification; mice survive to adulthood	*NR*
$Pax8^{-/-}TR\beta^{-/-}$	No TRβ Apo TRα Maximal Tshr signaling	*No Thyroid* T_4 undetectable T_3 undetectable TSH >400x	Growth retardation similar to $Pax8^{-/-}$; severely delayed endochondral ossification	Die by weaning
Deiodinase mutants				
C^3H/HeJ	80% reduction in D1 activity	T_4 1.6x T_3 normal rT_3 3x	*Not reported*	*NR*
$Dio1^{-/-}$	No *Dio1*	T_4 1.4x T_3 normal TSH normal rT_3 4x	Normal growth	*NR*
$Dio2^{-/-}$	No *Dio2*	T_4 1–1.3x T_3 normal TSH 3–15x rT_3 normal	Normal intramembranous and endochondral ossification	Reduced bone forr Increased mineraliz Brittle bones
$Dio3^{-/-}$	No *Dio3*	*Perinatal thyrotoxicosis* *Adult central hypothyroidism*	Reduced body length	*NR*
Compound mutants				
$Dio1^{-/-}$ $Dio2^{-/-}$	No *Dio1 or Dio2*	T_4 1.7x T_3 normal	Normal growth	*NR*

(*Continued*)

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Table 1. Continued

Mouse model	Genotype	Systemic thyroid status	Developing skeleton	Adult skeleton
		TSH 15x rT_3 6.5x		
Transporter mutants				
$Mct8^{-/-}$	No *Mct8*	T_4 0.7–0.3x T_3 1–1.4x TSH 1–3x rT_3 0.2x	*NR*	*NR*
$Mct10^{Y88*/Y88*}$	No *Mct10*	*P21:* T_4 normal; T_3 0.8x *Adult:* T_4 and T_3 normal	*NR*	*NR*
$Oatp1c1^{-/-}$	No *Oatp1c1*	T_4,T_3 and TSH normal	*NR*	*NR*
Compound mutants			*NR*	*NR*
$Mct8^{-/-}$ $Mct10^{Y88*/Y88*}$	No *Mct8 or Mct10*	*P21:* T_4 0.6x; T3 1.6x *Adult:* T_4 normal; T_3 3x	*NR*	*NR*
$Mct8^{-/-}$ $Oatp1c1^{-/-}$	No *Mct10 or Oatp1c1*	T_4 0.3x T_3 2x TSH 5x	Growth retarded after P16	*NR*
$Mct8^{-/-}$ $Dio1^{-/-}$	No *Mct8 or Dio1*	T_4 1.4x T_3 normal TSH 1.4x rT_3 normal	Mild growth retardation	*NR*
$Mct8^{-/-}$ $Dio2^{-/-}$	No *Mct8 or Dio2*	T_4 0.4x T_3 1.7x TSH 50x rT_3 0.2x	Mild growth retardation	*NR*
$Mct8^{-/-}$ $Dio1^{-/-}$ $Dio2^{-/-}$	No *Mct8, Dio1or Dio2*	T4 2.3x T_3 1.4x TSH 100x rT_3 2x	Growth retarded	*NR*

impaired linear growth, delayed endochondral ossification, reduced cortical bone mass, defective trabecular bone remodeling and reduced bone mineralization (138). Indeed, both *Pax8*$^{-/-}$ and hyt/hyt mice have impaired chondrocyte, osteoblast and osteoclast activities that are typical of thyroid hormone deficiency (161, 187, 189, 200, 230, 231, 246) and characteristic of juvenile hypothyroidism (278–281, 285). Nevertheless, the actions of thyroid hormone and TSH are not mutually exclusive, and the skeletal consequences of grossly abnormal thyroid hormone levels in *Pax8*$^{-/-}$ and hyt/hyt mice may mask effects of TSH on the skeleton.

To investigate further the role of the TSHR in bone, *Gpb5*$^{-/-}$ mice lacking the high affinity ligand thyrostimulin were characterized. Juvenile *Gpb5*$^{-/-}$ mice had increased bone volume and mineralization due to increased osteoblastic bone formation, whereas no effects on linear growth or osteoclast function were identified. Resolution of these abnormalities by adulthood was consistent with transient postnatal expression of thyrostimulin in bone (139). Despite this, treatment of osteoblasts with thyrostimulin in vitro had no effect on cell proliferation, differentiation and signaling, suggesting that thyrostimulin acts via unknown cellular and molecular mechanisms to inhibit bone formation indirectly during skeletal development.

Adult bone maintenance

Two animal studies have investigated the therapeutic potential of TSH to inhibit bone turnover. Ovariectomized rats were treated with TSH at doses insufficient to alter circulating T3, T4 or TSH levels (145). Intermittent TSH treatment resulted in reduced bone resorption markers, but increased formation markers, together with a dose related increase in BMD. Bone volume, trabecular architecture and strength parameters were either preserved or improved although no dose relationship was evident. This osteoblastic response to TSH (145) contrasts with findings in *Tshr*$^{-/-}$ mice, which also displayed increased osteoblastic bone formation despite the absence of TSHR signaling (32). In further studies, intermittent treatment of ovariectomized rats or mice with similar concentrations of TSH was investigated by bone densitometry and micro-CT. In these studies TSH prevented bone loss and increased bone mass following ovariectomy (286). Furthermore, treatment of thyroidectomized and parathyroidectomized rats with intermittent TSH injections also suppressed bone resorption and stimulated bone formation resulting in increased bone volume and strength (287).

B. Targeting thyroid hormone transport and metabolism

Thyroid hormone transporters

Mct8$^{-/y}$ knockout mice have elevated T3 but decreased T4 levels and recapitulate the systemic thyroid abnormalities observed in Allan-Herndon-Dudley syndrome. *Mct8*$^{-/y}$ mice, however, do not display the neurological abnormalities and exhibit only minor growth delay before postnatal day P35, suggesting that other transporters such as OATP1c1 may compensate for lack of MCT8 in mice (288, 289). *Mct10* mutant mice harbor an ENU loss-of-function mutation and exhibit normal weight gain during

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growth (290, 291). Furthermore, mice lacking both MCT8 and MCT10 also showed no evidence of growth retardation (291), suggesting both transporters are dispensable in the skeleton. Similarly, *Oatp1c1*$^{-/-}$ knockout mice exhibited normal weight gain during growth (292) and had no evidence of growth retardation (293). However, double mutants lacking both *Mct8* and *Oatp1c1* had growth retardation from P16 (292), confirming redundancy among thyroid hormone transporters in the regulation of skeletal growth. Finally, mice lacking *Mct8* and *Dio1* or *Dio2* display mild growth retardation, while triple mutants lacking *Mct8*, *Dio1* and *Dio2* exhibit more severe growth delay (288), indicating cooperation between thyroid hormone transport and metabolism in vivo during linear growth.

Deiodinases

A minor and transient impairment of weight gain was reported in male *Dio2*$^{-/-}$ mice, whereas weight gain and growth were normal in *Dio1*$^{-/-}$ and DIO1-deficient C3H/HeJ mice, and in C3H/HeJ/*Dio2*$^{-/-}$ mutants with DIO1 and DIO2 deficiency (294–296).

The role of DIO2 in bone was investigated in *Dio2*$^{-/-}$ mice (60), which have mild pituitary resistance to T4 characterized by a 3-fold increase in TSH, a 27% increase in T4 and normal T3 levels (297, 298). Bone formation and linear growth were normal in *Dio2*$^{-/-}$ mice, indicating DIO2 does not have a major role during postnatal skeletal development. This is unexpected given studies in the chick embryonic growth plate indicating that DIO2 regulates the pace of chondrocyte proliferation and differentiation during early development (159). Although skeletal development is normal, adult *Dio2*$^{-/-}$ mice have reduced bone formation resulting in a generalized increase in bone mineralization and brittle bones. Target gene analysis demonstrated the phenotype results from reduced T3 production in osteoblasts (60).

Dio3$^{-/-}$ mice have severe growth retardation and increased perinatal mortality. At weaning they have a 35% reduction in body weight, which persists into adulthood (299). Interpretation of the phenotype, however, is complicated by the systemic effects of disrupted HPT axis maturation and altered thyroid status (300).

In summary, DIO1 has no role in the skeleton; DIO2 is essential for osteoblast function and the maintenance of adult bone structure and strength, while the role of DIO3 in bone remains to be determined.

C. Targeting TRα (Figure 7)

Analysis of TR-null, Pax8-null and TR-'knock-in' mice has provided further insight into the complexity of T3 actions and the relative roles of TR isoforms. Importantly, TRα1$^{-/-}$ and TRα2$^{-/-}$ mice have selective deletion of TRα1 or α2, TR$\alpha^{-/-}$ mice represent an incomplete deletion because the TR$\Delta\alpha$1 and TR$\Delta\alpha$2 isoforms are still expressed, whereas TR$\alpha^{0/0}$ mice represent a complete knockout lacking all *Thra* transcripts (73, 185, 253, 301–303).

TRα1$^{-/-}$ mice retain normal TRα2 mRNA expression (304) and have 30% lower T4 but normal T3 levels and 20% reduced TSH, indicating mild central hypothyroidism. TRα1$^{-/-}$ mice had normal weight gain and linear growth (304). TRα1$^{-/-}$TR$\beta^{-/-}$ double-null mice have 60-fold increases in T4 and T3 with 160-fold higher TSH and decreased GH and IGF-1. Juveniles had growth retardation, delayed endochondral ossification and decreased bone mineralization, and adults had reduced trabecular and cortical BMD (305–307).

Gene targeting to prevent TRα2 expression resulted in 3–5 fold and 6–10 fold overexpression of TRα1 mRNA in TRα2$^{+/-}$ and TRα2$^{-/-}$ mice, respectively (253). TRα2$^{-/-}$ mice had 25% lower levels of T4, 20% lower T3, but inappropriately normal TSH, indicating mild thyroid dysfunction. Juvenile TRα2$^{-/-}$ mice had normal linear growth, but adults had reduced trabecular BMD and cortical bone mass (253). Fusion of green fluorescent protein (GFP) to exon 9 of *Thra* in TRα1-GFP mice unexpectedly resulted in loss of TRα2 expression in homozygotes with only a 2.5 fold increase in TRα1 mRNA (308). Homozygous TRα1-GFP mice were euthyroid with no abnormalities of postnatal development or growth, suggesting the phenotype in TRα2$^{-/-}$ mice may result from abnormal overexpression of TRα1. TRα2$^{-/-}$TR$\beta^{-/-}$ double mutants have mild hypothyroidism with 30% reduction in T4, 20% decrease in T3 and normal TSH, resulting in transiently delayed weight gain (309).

TR$\alpha^{-/-}$ mice are markedly hypothyroid, have severely delayed bone development and die around weaning unless treated with T3 (73, 310, 311). The skeletal abnormalities include delayed endochondral ossification, disorganized growth plate architecture, impaired chondrocyte differentiation and reduced bone mineralization. TR$\alpha^{-/-}$TR$\beta^{-/-}$ double-null mice have 10-fold increases in T4 and T3 with 100-fold elevation of TSH and similarly display delayed endochondral ossification and growth retardation (310, 311).

TR$\alpha^{0/0}$ mice are euthyroid and have less severe skeletal abnormalities than TR$\alpha^{-/-}$ mutants. Juveniles display growth retardation, delayed endochondral ossification and reduced bone mineral deposition. Although delayed ossification and reduced mineral deposition were observed in juvenile TR$\alpha^{0/0}$ mice, adults had markedly increased bone mass resulting from a bone-remodeling defect (312). Deletion of both TRs in TR$\alpha^{0/0}$TR$\beta^{-/-}$ double-null mice resulted in a more severe phenotype of delayed

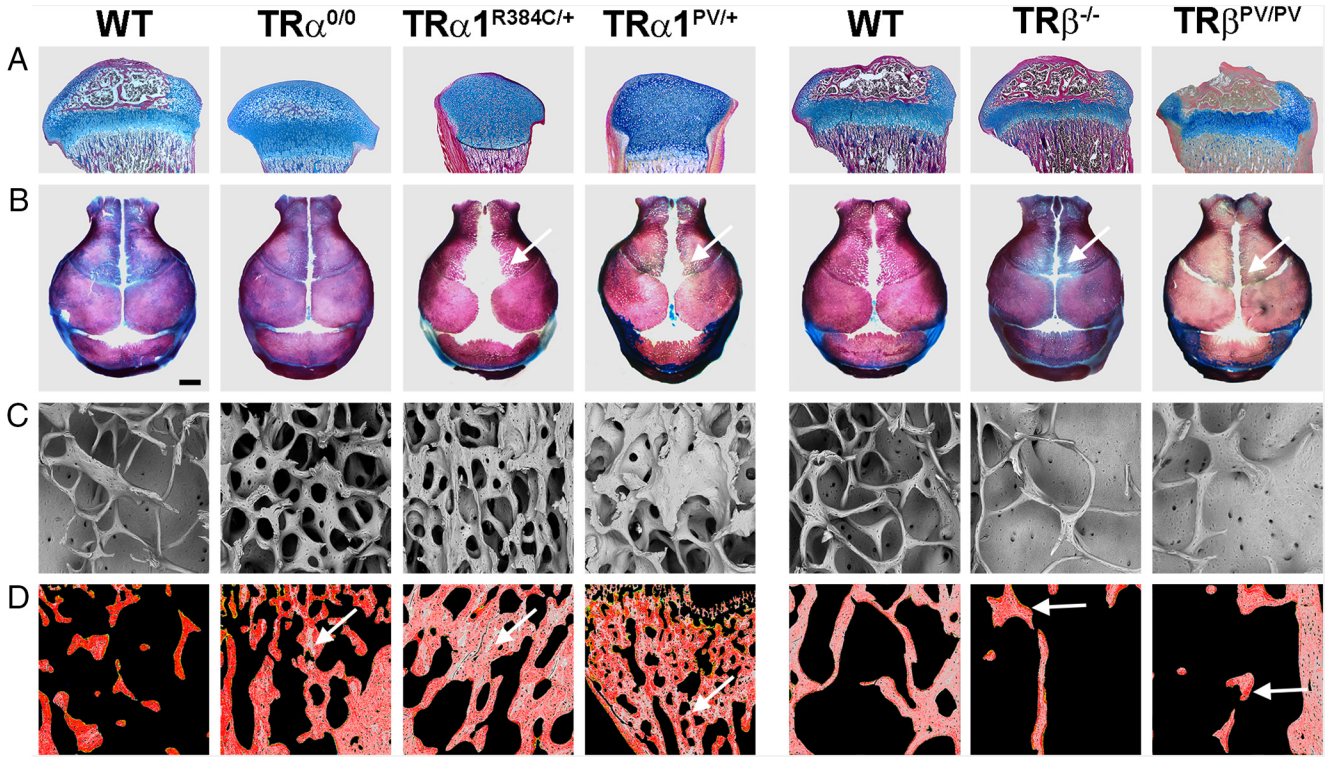
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bone maturation that may be due to reduced GH/IGF-I levels or reflect partial TRβ compensation in TR$\alpha^{0/0}$ single mutants (313).

The role of unliganded TRs in bone was studied in *Pax8*$^{-/-}$ mice. The severely delayed endochondral ossification in *Pax8*$^{-/-}$ mice compared to TR$\alpha^{0/0}$TR$\beta^{-/-}$ double mutants indicates that the presence of unliganded receptors is more detrimental to skeletal development than TR deficiency. Importantly, amelioration of the *Pax8*$^{-/-}$ skeletal phenotype in *Pax8*$^{-/-}$TR$\alpha^{0/0}$ knockout mice, but not *Pax8*$^{-/-}$TR$\beta^{-/-}$ mutants (314), suggested that unliganded TRα is largely responsible for the severity of the *Pax8*$^{-/-}$ skeletal phenotype. Nevertheless, this interpretation was not supported by analysis of *Pax8*$^{-/-}$TRα1$^{-/-}$ mice in which growth retardation in *Pax8*$^{-/-}$ mice was not ameliorated by additional deletion of TRα1 (315).

The essential role for TRα in bone has been confirmed by studies of mice with dominant negative mutations of *Thra1*. A patient with severe resistance to thyroid hormone (RTH) was found to have a frameshift mutation in the *THRB* gene, termed "PV". The mutation results in expression of a mutant TR that cannot bind T3 and acts

Figure 7.



Skeletal phenotype of TRα and TRβ mutant mice

(A) Proximal tibias stained with alcian blue (cartilage) and van Gieson (bone, red) showing delayed formation of the secondary ossification center in TRα deficient mice (TR$\alpha^{0/0}$) and grossly delayed formation in mice with dominant negative TRα mutations (TRα1$^{R384C/+}$ and TRα1$^{PV/+}$). Mice with mutation or deletion of TRβ have advanced ossification with premature growth plate narrowing.

(B) Skull vaults stained with alizarin red (bone) and alcian blue (cartilage) showing skull sutures and fontanelles. Arrows indicate delayed intramembranous ossification in mice with dominant negative TRα mutations (TRα1$^{R384C/+}$ and TRα1$^{PV/+}$) and advanced ossification in mice with mutation or deletion of TRβ.

(C) Trabecular bone microarchitecture in adult TR mutant mice. Backscattered electron scanning electron microscopy (EM) images show increased trabecular bone in TR$\alpha^{0/0}$ mice and severe osteosclerosis in TRα1$^{R384C/+}$ and TRα1$^{PV/+}$ mice. By contrast, TRβ mutant mice have reduced trabecular bone volume and osteoporosis.

(**D)** Trabecular bone micromineralization in adult TR mutant mice. Pseudocolored quantitative backscattered electron scanning EM images showing mineralization densities in which high mineralization density is gray and low is red. Mice with deletion or mutation of TRα have retention of highly mineralized calcified cartilage (arrows) demonstrating a persistent remodeling defect. By contrast, mice with deletion or mutation of TRβ have reduced bone mineralization (arrow) secondary to increased bone turnover.

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as a potent dominant-negative antagonist of wild-type TR function (316). The homologous mutation was introduced into the mouse *Thra* gene to generate TRα1^{PV} mice. The TRα1$^{PV/PV}$ homozygous mutation is lethal whereas TRα1$^{PV/+}$ heterozygotes display mild thyroid failure with small increases in TSH and T3, but no change in T4. TRα1$^{PV/+}$ mice have a severe skeletal phenotype with growth retardation, delayed intramembranous and endochondral ossification, impaired chondrocyte differentiation and reduced mineral deposition during growth (278, 317, 318). Adult mice had grossly abnormal skeletal morphology with increased bone mass and retention of calcified cartilage indicating defective bone remodeling (319). Thus, TRα1$^{PV/+}$ mice have markedly impaired bone development and maintenance despite systemic euthyroidism, indicating that wild-type TRα signaling in bone is impaired by the dominant-negative PV mutation in heterozygous mice. Furthermore, the presence of a dominant-negative TRα leads to a more severe phenotype than receptor deficiency alone.

TRα1$^{R384C/+}$ mice, with a less potent dominant-negative mutation of TRα, are euthyroid. They display transient growth retardation with delayed intramembranous and endochondral ossification. Trabecular bone mass increased progressively with age and adults had osteosclerosis due to a remodeling defect (312). Remarkably, brief T3 supplementation during growth, at a dose sufficient to overcome transcriptional repression by TRα1^{R384C}, ameliorated the adult phenotype (312). Thus, even transient relief from transcriptional repression mediated by unliganded TRα1 during development has long-term consequences for adult bone structure and mineralization.

TRα^{AMI} mice harbor a floxed *Thra* allele [AF2 mutation inducible (AMI)] and express a dominant-negative mutant TRα1^{L400R} only after Cre-mediated recombination. Mice with global expression of TRα1^{L400R} had a similar skeletal phenotype to TRα1$^{PV/+}$ and TRα1$^{R384C/+}$ mice (320). Furthermore, restricted expression of TRα1^{L400R} in chondrocytes resulted in delayed endochondral ossification, impaired linear growth and a skull base defect (321). Microarray studies using chondrocyte RNA revealed changes in expression of known T3-regulated genes, but also identified new target genes associated with cytoskeleton regulation, the primary cilium, and cell adhesion. Desjardin et al also reported that restricted expression of TRα1^{L400R} in osteoblasts unexpectedly resulted in no skeletal abnormalities (321).

In summary, the presence of an unliganded or mutant TRα is more detrimental to the skeleton than the absence of the receptor. Overall, these studies (i) demonstrate the importance of thyroid hormone signaling for normal skeletal development and adult bone maintenance, and (ii) identify a critical role for TRα in bone.

D. Targeting TRβ (Figure 7)

Two TR$\beta^{-/-}$ strains have been generated and both show similar skeletal phenotypes (9, 285, 311–313). Mice lacking TRβ recapitulate RTH with increased T4, T3 and TSH concentrations (9, 285). Juvenile TR$\beta^{-/-}$ mice have accelerated endochondral and intramembranous ossification, advanced bone age, increased mineral deposition and persistent short stature due to premature growth plate closure. Increased T3 target gene expression demonstrated enhanced T3 action in skeletal cells resulting from the effects of elevated thyroid hormones mediated by TRα in bone. Accordingly, the phenotype is typical of the skeletal consequences of thyrotoxicosis (13, 322). In keeping with the consequences of thyrotoxicosis, adult TR$\beta^{-/-}$ mice have progressive osteoporosis with reduced trabecular and cortical bone, reduced mineralization and increased osteoclast numbers and activity (285, 312).

TR$\beta^{PV/PV}$ mice with the potent dominant-negative PV mutation have a severe RTH phenotype with a 15-fold increase in T4, 9-fold increase in T3 and 400-fold increase in TSH (164, 278, 317, 323). Consequently, TR$\beta^{PV/PV}$ mice have more severe abnormalities than TR$\beta^{-/-}$ mice with accelerated intrauterine growth, advanced endochondral and intramembranous ossification, craniosynostosis and increased mineral deposition again resulting in short stature.

Overall, skeletal abnormalities in TRβ mutant mice are also consistent with a predominant physiological role for TRα in bone.

Cellular and molecular mechanisms

The opposing skeletal phenotypes in mutant mice provide compelling evidence of distinct roles for TRα and TRβ in the skeleton and HPT axis. The contrasting phenotypes of TRα and TRβ mutant mice can be explained by the predominant expression of TRα in bone (163, 164) and TRβ in hypothalamus and pituitary (8, 9). Thus, in TRα mutants, delayed ossification with impaired bone remodeling in juveniles and increased bone mass in adults is a consequence of disrupted T3 action in skeletal T3 target cells, whereas accelerated skeletal development and adult osteoporosis in TRβ mutants result from supraphysiological stimulation of skeletal TRα due to disruption of the HPT axis (318).

This paradigm is supported by analysis of T3 target gene expression in skeletal cells, which demonstrated reduced skeletal T3 action in TRα mutant animals (255, 278, 285, 312), and increased T3 action in TRβ mutant mice (164, 224, 285, 312). The studies further demon-

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strate that T3 exerts anabolic actions during skeletal development but catabolic actions in adult bone. The data also indicate that effects of disrupted or increased T3 action in bone predominate over skeletal responses to TSH. For example, TR$\alpha^{0/0}$, TR$\alpha1^{PV/+}$ and TR$\alpha1^{R384C/+}$ mice have grossly increased trabecular bone mass even when TSH concentrations are normal, while TR$\beta^{-/-}$ and TR$\beta^{PV/PV}$ mice are osteoporotic despite markedly increased levels of TSH. Consistent with this conclusion, TR$\alpha1^{-/-}\beta^{-/-}$ double knockout mice, generated in a different genetic background, also display reduced bone mineralization despite grossly elevated TSH (305).

Although TRα is the major TR isoform expressed in bone, it is apparent TR$\alpha^{0/0}$TR$\beta^{-/-}$ mice have a more severe skeletal phenotype than TR$\alpha^{0/0}$ mice, while TR$\alpha^{0/0}$ mice also remain sensitive to T4 treatment, suggesting a compensatory role for TRβ in skeletal cells. This analysis is supported by studies of thyroid manipulated adult TR$\alpha^{0/0}$ and TR$\beta^{-/-}$ mice, in which a discrete role for TRβ in mediating remodeling responses to T3 treatment was proposed (167).

Downstream signaling responses in skeletal cells

GH receptor (GHR) and IGF1 receptor (IGF1R) mRNA was reduced in growth plate chondrocytes in TR$\alpha^{0/0}$ and TR$\alpha1^{PV/+}$ mice and phosphorylation of their secondary messengers signal transducer and activator of transcription 5 (STAT5) and protein kinase B (AKT) was also impaired (278, 312). By contrast, GHR and IFG1R expression and downstream signaling were increased in TR$\beta^{-/-}$ and TR$\beta^{PV/PV}$ mice (278, 285). Furthermore, studies in osteoblasts from knockout mice revealed that T3/TRα1 bound to a response element in intron 1 of the Igf1 gene to stimulate transcription (259). These studies demonstrate GH/IGF1 signaling is a downstream mediator of T3 action in the skeleton in vivo.

Activation of FGFR1 stimulates osteoblast proliferation and differentiation (324) and FGFR3 regulates growth plate chondrocyte maturation and linear growth (325). *Fgfr3* and *Fgfr1* expression was reduced in growth plates of TR$\alpha^{0/0}$, TR$\alpha1^{R384C/+}$ and TR$\alpha1^{PV/+}$ mice and *Fgfr1* expression was reduced in osteoblasts from TR$\alpha^{0/0}$ and Pax8$^{-/-}$ mice (138, 224, 255, 278, 285, 312). By contrast, *Fgfr3* and *Fgfr1* expression was increased in growth plates of TR$\beta^{-/-}$ and TR$\beta^{PV/PV}$ mice and *Fgfr1* expression was increased in osteoblasts from TR$\beta^{PV/PV}$ mice (164, 224, 285, 312). Thus, FGF/FGFR signaling is a downstream mediator of T3 action in chondrocytes and osteoblasts in vivo.

T3 is essential for cartilage matrix synthesis and heparan sulfate proteoglycans (HSPGs) are key matrix components essential for FGF and IHH signaling. Studies in thyroid manipulated rats and TR$\alpha^{0/0}$TR$\beta^{-/-}$ and Pax8$^{-/-}$ mice demonstrated reduced HSPG expression in thyrotoxic animals, increased expression in TR$\alpha^{0/0}$TR$\beta^{-/-}$ mice and more markedly increased expression in hypothyroid rats and congenitally hypothyroid Pax8$^{-/-}$ mice (219). Thus, T3 coordinately regulates FGF/FGFR and IHH/PTHrP signaling within the growth plate via regulation of HSPG synthesis.

In neonatal TR$\beta^{PV/PV}$ mice, *Runx2* expression was increased in perichondrial cells surrounding the developing growth plate, and in 2-week-old mice *Rankl* expression was decreased in osteoblasts (252). Although the findings are consistent with increased canonical Wnt signaling pathway during postnatal growth, expression of Wnt4 was decreased. Unliganded TRβ physically interacts with and stabilizes β-catenin to increase Wnt signaling but this interaction is disrupted by T3 binding. However, although TRβ^{PV} similarly interacts with β-catenin its interaction is not disrupted by T3 (326), and this leads to persistent activation of Wnt signaling in TR$\beta^{PV/PV}$ mice despite reduced expression of Wnt4 (252). Tsourdi et al investigated Wnt signaling in hypothyroid and thyrotoxic adult mice (327). In hyperthyroid animals bone turnover was increased and, consistent with this, the serum concentration of the Wnt inhibitor DKK1 was decreased. In hypothyroid mice bone turnover was reduced and the DKK1 concentration increased. Surprisingly, however, concentrations of another Wnt inhibitor, sclerostin, were increased in both hyperthyroid and hypothyroid mice (327). These preliminary studies suggest increased Wnt signaling may also lie downstream of T3 action in bone.

VI. Skeletal consequences of mutations in thyroid signaling genes in humans (Table 2)

A. TSHB

Loss of function mutations

Several individuals have been described with mutations of *TSHB* leading to biologically inactive TSH and congenital nongoitrous hypothyroidism (OMIM #275100), but skeletal consequences have only been documented in two cases. Two brothers aged 10 and 7 years were described with isolated TSHβ deficiency and normal BMD following thyroid hormone replacement from birth (328). These cases demonstrate that absence of TSH throughout normal skeletal development and growth does not affect bone mineral accumulation by 10 years of age.

B. TSHR

Loss-of-function mutations

Loss-of-function mutations in *TSHR* have been described at more than 30 different amino acid positions and

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Table 2. Skeletal phenotype in individuals with *TSHB*, *TSHR*, *SECISBP2*, *THRA* and *THRB* mutations

Mutation	Genotype	Systemic thyroid status	Skeletal Phenotype	References
***TSHB* loss-of-function (Non-goitrous congenital hypothyroidism type 4 OMIM: 275 100)**				
$TSHB^{Q49X/Q49X}$	Premature stop TSH deficiency	Low T4, T3 and TSH	T4 replacement from birth BMD normal in two children at 7y and 10y	(328)
***TSHR* loss-of-function (Non-goitrous congenital hypothyroidism type 1 OMIM: 275 200)**				
$TSHR^{P52A/I167N}$	(i) Impaired function (ii) Severely impaired function	Normal T4 Elevated TSH 20x	Normal growth and bone age (14y) *(Without T4 treatment)*	(334)
$TSHR^{R109Q/W546X}$	(i) Impaired function (ii) frameshift/premature stop	Normal T4 Elevated TSH × 50	Normal growth and bone maturation (3y) *(Without T4 treatment)*	(333)
$TSHR^{A553T/A553T}$	Reduced cell surface expression	Hypoplastic thyroid Low T4 Elevated TSH × 250	Normal birth length and head circumference Normal growth (3y) *(T4 treatment)*	(331)
$TSHR^{C390W/\ fs419X}$	(i) Impaired function (ii) frameshift/premature stop	Hypoplastic thyroid Low T4 Elevated TSH 40x	Normal linear growth and bone age (5y) *(T4 treatment)*	(332)
$TSHR^{IVS6\ +\ 3\ G>C/fs656X}$	(i) Skipping of exon 6 (ii) frameshift/premature stop	Severe hypoplasia Very low T4 Elevated TSH 200x	Delayed bone age/enlarged fontanelles at birth Normal growth (2y) *(T4 replacement)*	(336)
$TSHR^{IVS5–1G>A/IVS5–1G>A}$	Skipping of exon 6	Severe hypoplasia Very low T4 and T3 Elevated TSH 250x	Enlarge fontanelles at birth Normal growth (12y) *(T4 replacement)*	(335)
***TSHR* gain-of-function (Non-autoimmune hyperthyroidism OMIM: 609 152)**				
$TSHR^{M453T/+}$	Constitutive activation of cAMP	Goiter High T4 and T3 Suppressed TSH	Advanced bone age Normalised after treatment (12y)	(539)
$TSHR^{M453T/+}$	Constitutive activation of cAMP	Goiter High T4 and T3 Suppressed TSH	Advanced bone age (newborn)	(339)
$TSHR^{S505N/+}$	Constitutive activation of cAMP	High T4 and T3 Suppressed TSH	Bone age advanced by 4 yr at 6 moths old Craniosynostosis Treatment improved growth and bone age (4y)	(341)
$TSHR^{T632I/+}$	Constitutive activation of cAMP	High T4 and T3 Suppressed TSH	Craniosynostosis	(344)
$TSHR^{F629L/+}$	Constitutive activation	Goiter High T4 and T3 Suppressed TSH	Advanced bone age Craniosynostosis Development normal after treatment (10y)	(338)
$TSHR^{R528H/S281N}$	(i) Polymorphism (ii) Constitutive activation of cAMP	High T4 and T3 Suppressed TSH	Birth length 90^{th} centile Craniosynostosis requiring surgery	(343)
$TSHR^{V597L/+}$	Constitutive activation of cAMP	High T4 and T3 Suppressed TSH	Advanced bone age (9m)	(340)
$TSHR^{M453T/+}$	Constitutive activation of cAMP	Goiter High T4 and T3 Suppressed TSH	Bone age advanced by 5y Craniosynostosis and mid-face hypoplasia Shortened 5^{th} metacarpals and phalanges	(342)
$TSHR^{T32I/+}$	Constitutive activation	High T4 and T3 Suppressed TSH	Bone age advanced by 4 yr Craniosynostosis requiring surgery Treatment stabilized bone age (Height: 90^{th} centile at 6months but 18^{th} centile at 4y)	(345)
***SECISBP2* loss-of-function (Abnormal thyroid hormone metabolism OMIM: 609 698)**				
$SECISBP2^{R540Q/R540Q}$	Hypomorphic allele with abnormal SECIS binding	High T4 and rT3, low T3, Slightly elevated TSH;	Delayed growth and bone age Normal final height	(346)
$SECISBP2^{K438X/IVS8DS+29G-A}$	(i) LoF truncation (ii) Splicing defect	High T4 and rT3, low T3, Slightly elevated TSH	Transient growth retardation	(346)
$SECISBP2^{R128X/R128X}$	Truncated protein lacking N-terminal	High T4 and rT3 low T3 normal TSH.	Growth retardation and delayed bone age. Responsive to T3 treatment	(348)
$SECISBP2^{R120X/R770X}$	(i) LoF truncation (ii) Impaired SECIS binding	High T4 and rT3 low T3 Slightly elevated TSH	Intrauterine growth retardation Craniofacial dysmorphism Bilateral clinodactyly Delayed growth and bone age	(347)
$SECISBP2^{fs255X/\ fs}$	(i) frameshift/premature stop (ii) splicing defect	High T4 and rT3 normal T3 normal TSH	Genu valgus External rotation of the hip Normal final height	(349)
$SECISBP2^{C691R/fs}$	(i) Increased proteasomal degradation (ii) splicing defect	High T4 and rT3 low T3 normal TSH	Delayed growth T3 treatment improved growth	(349)
$SECISBP2^{M515fs563X/Q79X}$	(i) frameshift/premature stop (ii) premature stop	High T4 slightly low T3 Slightly elevated TSH	Delayed growth and bone age GH improved height but not bone age GH+T3 normalised height and bone age	(350)
RTHβ: *THRB* dominant-negative mutations (Nongoitrous congenital hypothyroidism type 6 OMIM: 614 450)				
$TR\beta^{?/+}$	Unknown	Goitre High T4, T3 Non suppressed TSH	Delayed bone maturation Stippled epiphyses	(351)
$TR\beta^{?/+}$	Unknown	Goiter High T4, T3 Non suppressed TSH	Normal height and body proportions Normal head circumference Normal bone age and epiphyses (8y)	(360)
$TR\beta^{?/+}$	Unknown	High T4, T3 and TSH	Short stature and delayed bone age.	(540)
$TR\beta^{?/+}$	Unknown	Goiter High T4, T3 Non suppressed TSH	Short stature Bone age 3y at 2.9 yr old Craniosynostosis (9y) Shortened 4th metacarpals and metatarsals	(377)

(*Continued*)

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Table 2. Continued

Mutation	Genotype	Systemic thyroid status	Skeletal Phenotype	References
TRβ$^{G345R/+}$	No T3 binding	Goiter High T4, T3 Non suppressed TSH	Normalizing T4 slowed growth	(352)
TRβ$^{\Delta T337/\Delta T337}$	Dominant negative TRβ Homozygous single amino acid deletion No T3 binding	High T4, T3 Markedly elevated TSH	Growth retardation Delayed bone age	(368,370)
TRβ$^{-/-}$	Deletion of TRβ coding region	High T4, T3 Non suppressed TSH	Stippled epiphyses and growth delay	(541)
TRβ$^{R438H/+}$	Dominant negative TRβ Missense mutation	Goiter High T4 and TSH	Delayed bone age.	(542)
TRβ$^{C434X/+}$	Dominant negative TRβ C-terminal premature stop No T3 binding	High T4, T3 Non suppressed TSH	Advanced bone age	(376)
TRβ$^{R338L/+}$	Dominant negative TRβ Missense mutation	High T4, T3 Non suppressed TSH	Reduced bone mineral density	(375)
TRβ$^{M310L/+}$	Dominant negative TRβ Missense mutation Reduced T3 affinity	Goiter High T4, T3 Non suppressed TSH	Intrauterine growth retardation Delayed bone age Persistent short stature (17y)	(366)
TRβ$^{F438fs422X/+}$	Potent dominant negative TRβ Frameshift C-terminal premature stop No T3 binding	Small goiter Very high T4, T3 High TSH	Growth retardation and delayed bone age Short stature Frontal bossing	(367)
TRβ$^{K443fs458X/+}$	Potent dominant negative TRβ Frameshift C-terminal premature stop No T3 binding	High T4, T3 and TSH	Short stature (7y)	(316)
TRβ$^{R429W/+}$	Normal T3-binding affinity and transactivation	Goitre High T4, T3 Non suppressed TSH	Increased osteocalcin and decreased BMD (19y)	(378)
TRβ$^{G344A/+}$	Dominant negative TRβ Missense mutation No T3 binding	High T4 Non suppressed TSH	Mild intrauterine growth retardation Persistent short stature Delayed bone age	(364,543)
TRβ$^{G347A/+}$	Missense mutation	Goiter High T4, T3 Non suppressed TSH	Persistent short stature (21y) Increased bone turnover markers	(379)
TRβ$^{A317T/+}$; TRβ$^{R438H/+}$; TRβ$^{P453t/+}$; TRβ$^{P453fs463X/+}$; TRβ$^{N331H/+}$	Missense mutations	High T4, T3	Reduced whole body and lumbar spine BMD	(374)
	Frameshift C-terminal premature stop	Non suppressed or high TSH	in adults but not in children	
RTHα: *THRA* dominant-negative mutations (Nongoitrous congenital hypothyroidism type 6 OMIM: 614 450)				
TRα1$^{E403X/+}$	Dominant negative TRα1 C-terminal premature stop	Low fT4, normal fT3 Low fT4/fT3 ratio Low rT3 Normal TSH	Disproportionate short stature (6y) Epiphyseal dysgenesis and grossly delayed bone age Macrocephaly and patent skull sutures Delayed tooth eruption, defective bone mineralization	(382)
TRα1$^{F397fs406X/+}$	Dominant negative TRα1 Frameshift C-terminal premature stop	Low fT4 and high fT3 Low fT4/fT3 ratio Low rT3 Normal TSH	Delayed bone age and persistent short stature (3y) Macrocephaly/flattened nasal bridge/patent skull sutures Delayed tooth eruption and congenital hip dislocation Brief increased growth following T4 treatment Persistent short stature and otosclerosis (47y)	(384,385)
TRα1$^{A382Pfs389X/+}$	Dominant negative TRα1 Frameshift C-terminal premature stop	Normal fT4 and fT3 Low fT4/fT3 ratio Low rT3 Borderline elevated TSH	Disproportionate persistent short stature (45y) Macrocephaly and cortical bone thickening T4 treatment (10–15y) increased growth rate	(383)
TRα1$^{C392X/+}$	C-terminal premature stop	Low fT4 High fT3 Low fT4/fT3 ratio Normal TSH	Delayed growth short stature with shortened limbs Macrocephaly/flattened nasal bridge/hypertelorism Micrognathia/elongated thorax/lumbar kyphosis (18y) T4 treatment did not improve syndrome	(388)
TRα1$^{E403X/+}$	Dominant negative TRα1 C-terminal premature stop	Low normal fT4 High normal fT3 Low fT4/fT3 ratio Normal TSH	Delayed growth short stature with shortened limbs Macrocephaly/flattened nasal bridge/hypertelorism Elongated thorax/lumbar kyphosis (14y) T4 treatment did not improve syndrome	(388)
TRα1$^{E403K/+}$	Missense mutation	Low normal fT4 High normal fT3 Low fT4/fT3 ratio Normal TSH	Short stature with shortened limbs Macrocephaly/flattened nasal bridge/hypertelorism Elongated thorax (12y) T4 treatment did not improve syndrome	(388)
TRα1$^{P398R/+}$	Missense mutation	Low normal fT4 High normal fT3 Low fT4/fT3 ratio Low normal TSH	Delayed growth with shortened limbs Flattened nasal bridge/hypertelorism Elongated thorax (8y) T4 treatment did not improve syndrome	(388)
TRα$^{A263V/+}$	Weak dominant negative Missense mutation Transactivation activity restored by supra-physiological T3 No effect on TRα2 action	Low normal fT4 High normal fT3 Low fT4/fT3 ratio Low rT3 Normal TSH	Growth retardation, Macrocephaly/broad face/flattened nasal bridge Thickened calvarium T4 treatment improved growth rate Macrocephaly and increased BMD in adult	(389)
TRα$^{N359Y/+}$	Dominant-negative TRα1 Missense mutation with decreased T3 binding and transactivation activity	Normal fT4	Growth retardation with shortened limbs	(390)

(*Continued*)

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Table 2. Continued

Mutation	Genotype	Systemic thyroid status	Skeletal Phenotype	References
	Weak reduction in TRα2 action	High normal fT3 Low fT4/fT3 ratio	Macrocephaly/hypertelorism/micrognathia/broad nose, Elongated thorax with clavicular and12th rib agenesis with Scoliosis, ovoid vertebrae and congenital hip dislocation	
		Low normal rT3 Low TSH (initially normal)	Humeroradial synostosis and syndactyly	

result in varying degrees of TSH resistance and congenital nongoitrous hypothyroidism (OMIM #275200) (329, 330). However, skeletal consequences have only been reported in a few children. Individuals with mild and compensated hypothyroidism have normal growth and bone age (331–334), whereas subjects with severe thyroid hypoplasia and very low T4 and T3 levels have delayed intramembranous ossification and bone age at birth but display normal growth and postnatal skeletal development following T4 replacement (335, 336). These cases demonstrate that impaired TSHR signaling does not impair linear growth and bone maturation when thyroid hormones are adequately replaced.

Gain-of-function mutations

Nonautoimmune autosomal dominant hyperthyroidism (OMIM #609152) is rare and patients are frequently treated at an early age with thyroid surgery and radioiodine ablation followed by physiological T4 replacement (337). Skeletal manifestations at presentation include classical features of juvenile hyperthyroidism, with advanced bone age (338–342) craniosynostosis (338, 342–345), shortening of fifth metacarpal bones and middle phalanges of the fifth fingers (342) all described. The skeletal consequences in treated adults have not been reported, but in several younger patients amelioration of the phenotype was reported following treatment and normalization of circulating thyroid hormone levels (338, 341, 344, 345). These cases indicate that early normalization of thyroid status improves skeletal abnormalities in patients with nonautoimmune autosomal dominant hyperthyroidism despite continued constitutive activation of the TSHR.

C. SBP2

Loss-of-function mutations

The deiodinases are selenoproteins that require incorporation of the rare amino acid selenocysteine (Sec) for enzyme activity. Individuals with loss-of-function mutations of *SBP2*, which encodes the Sec incorporation sequence binding protein (SBP2) essential for incorporation of Sec, have abnormal thyroid hormone metabolism resulting in low circulating total and fT3 levels despite elevated total and fT4 and reverse T3 levels (OMIM #609698). Affected patients have evidence of skeletal thyroid hormone deficiency during prepubertal growth with transient growth retardation, short stature and delayed bone age (346–349) that cannot be compensated by treatment with GH (350).

D. THRB

Dominant-negative mutations

Resistance to thyroid hormone (RTH), an autosomal dominant condition caused by heterozygous dominant negative mutations of the *THRB* gene encoding TRβ (OMIM #188570, RTHβ), was described in 1967 (351) with the first mutations identified in 1989 (352, 353). The incidence of RTHβ is 1 in 40 000 and over 3000 cases have been documented with 80% harboring *THRB* mutations, of which 27% are de novo (354). The mutant TRβ disrupts negative feedback of TSH secretion resulting in the characteristic elevation of T4 and T3 concentrations and inappropriately normal or increased TSH. The syndrome results in a complex mixed phenotype of hyperthyroidism and hypothyroidism depending on the specific mutation and target tissue. Thus, an individual patient can have symptoms and signs of both thyroid hormone deficiency and excess. Tissue responses to T3 are dependent upon: the severity of the TRβ mutation; genetic background; the relative concentrations of TRα, wild-type TRβ and dominant negative mutant TRβ proteins in individual cells; and whether the patient has received prior treatment with antithyroid drugs or surgery. Consequently, interpretation of the skeletal phenotype in RTHβ is complex and a broad range of abnormalities has been described.

The skeletal consequences of RTHβ have been reported in a limited number of patients with a spectrum of mutations. Abnormalities include major or minor somatic defects, such as scaphocephaly, craniosynostosis, bird-like facies, vertebral anomalies, pigeon breast, winged scapulae, prominent pectoralis, and short fourth metacarpals (355). The findings are inconsistent and factors other than mutations of TRβ may be responsible (356). In kindreds with the same *THRB* mutation, individuals may have different phenotypes especially with respect to growth velocity or the degree of abnormality in thyroid status. Thus,

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variable skeletal abnormalities have been reported in 3 kindreds (357), in 14 affected individuals within a four-generation kindred (358), and in four unrelated families with affected individuals aged 14 months to 29 years (359), while a normal skeletal phenotype was reported in an affected 8-year-old (360).

Some subjects have been reported with skeletal abnormalities that are similar to the consequences of hypothyroidism. Delayed growth <fifth percentile has been estimated in 20% of subjects with growth retardation alone in 4% (361), while delayed bone age of > 2 SD was reported to occur in 29%–47% (362). An NIH study of 104 patients from 42 kindreds and 114 unaffected relatives found short stature in 18%, low weight-for-height in 32% of children, and variable tissue resistance from kindred to kindred. RTHβ patients were shorter than unaffected family members and had no catch-up growth later in life. However, delayed bone age was documented in only a minority (363). In a study of 36 family members with RTHβ in four generations, delayed bone maturation with short stature was observed in affected adults and children. Of note, in affected individuals born to an affected mother, growth retardation was less marked. Seven of eight children with RTHβ, but only one of six controls, had bone age 2SD below mean (364). Delayed bone maturation was also found in a series of 8 children (365). In individual cases stippled epiphyses were reported in a 6 year old (351), intra-uterine and postnatal growth retardation with delayed bone age in a 26-month old girl (366), and delayed bone age > 3SD below mean for chronological age in a 22 month-old (367). Four patients with homozygous *THRB* mutations have been identified and markedly delayed linear growth and skeletal maturation were reported (368–370).

Other individuals with RTHβ, however, have been reported with skeletal abnormalities that are similar to the consequences of hyperthyroidism. Short metacarpals, metatarsals and advanced bone age have all been described (371), as well as craniofacial abnormalities, craniosynostosis, advanced bone age, short stature, osteoporosis and fracture, together with increased bone turnover and changes in calcium, phosphorous and magnesium metabolism (364, 372, 373). In 14 patients, including eight adults and six children, the affected children had short stature below the third centile, no significant delay in bone age, increased calcium, decreased phosphate and elevated FGF23. Adults had low lumbar spine and whole body BMD (374). Five adults from two unrelated kindreds with same *THRB* mutation were followed for 3–11 years and found to have reduced BMD (375). In individual cases, a 15 year old girl with short stature and advanced bone age equivalent to age 17 years (376) was reported; craniosynostosis with frontal bossing and minor skeletal abnormalities including short metacarpals were seen in a 9-year-old (377); a 19-year-old man with increased osteocalcin and mildly reduced BMD was reported (378); and a 21-year-old female with short stature, normal alkaline phosphatase but elevated urinary deoxypyridinoline was described (379).

Overall, these findings should be considered in the context of data from mouse models of RTHβ. In mutant mice, the skeletal phenotype is consistent with increased thyroid hormone action in bone manifest by accelerated skeletal development in juveniles and increased bone turnover with osteoporosis in adults (318). Importantly, the clear and consistent findings in mutant mice result from studies of single mutations in genetically homogeneous backgrounds that are not confounded by therapeutic intervention. Reports in human RTHβ result from patients with a broad range of mutations of varying severity studied in diverse genetic backgrounds. Phenotype diversity is also likely to be due to additional confounding factors including; variable phenotype analyses and nomenclature among studies, retrospective and cross-sectional study design, differing surgical and pharmacological interventions, and analysis of individuals at differing ages. Well-controlled long-term prospective analysis of large families with specific mutations will be the only way to determine the skeletal consequences of individual mutations in human RTHβ.

E. THRA

Dominant-negative mutations

Individuals with mutations of *THRA* encoding TRα were recently identified (OMIM #614450, RTHα) and this has resulted in reclassification of the inheritable syndromes of impaired sensitivity to thyroid hormone (380). By analogy to the phenotype variability seen in RTHβ, it was considered likely that individuals with a spectrum of *THRA* mutations would be identified (381). Heterozygous mutations of *THRA* were first reported in three families in 2012 and 2013 (382–385). Affected individuals all had grossly delayed skeletal development but variable motor and cognitive abnormalities. All had normal serum TSH with low/normal T4 and high/normal T3 concentrations, and a characteristically reduced fT4:fT3 ratio (369, 386, 387).

Mutations affecting TRα1

A 6-year-old girl had skeletal dysplasia, growth retardation with grossly delayed bone age and tooth eruption, patent skull sutures with wormian bones, macrocephaly, flattened nasal bridge, disproportionate short stature (de-

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creased subischial leg length with a normal sitting height), epiphyseal dysgenesis and defective bone mineralization (382). A 3-year-old girl displayed a similar phenotype with short stature, macrocephaly, delayed closure of the skull sutures, delayed tooth eruption, delayed bone age with absent hip secondary ossification centers and congenital hip dislocation. Treatment with T4 resulted in a brief initial catch-up of growth, whereas GH treatment had no effect. Her subsequent height, however, remained 2 SD below normal. Her 47-year-old father was short (-3.77 SD) but with normal BMD and he had hearing loss due to otosclerosis (384, 385). A 45-year-old female was subsequently described with disproportionate short stature and macrocephaly (head circumference +9 SD), together with skull vault and long-bone cortical thickening. Between the ages of 10 and 15 years, T4 treatment increased her rate of growth, although final adult height was 2.34 SD below predicted (383). Most recently, a series of four individuals with truncating and missense mutations affecting TRα1 was described and included data from 18 years follow-up (388). A consistent skeletal phenotype included disproportionate growth retardation with relatively short limbs, hands and feet and a long thorax, and a mild skeletal dysplasia comprising flat nasal bridge with round, puffy and coarse flattened facial features, upturned nose, hypertelorism and macrocephaly. Radiological features of hypothyroidism included: ovoid immature vertebral bodies, ossification defects of lower thoracic and upper lumbar bodies and hypoplasia of the acetabular and supra-acetabular portions of the ilia, and coxa vara. Hand X-rays showed changes in the tubular bones, which were abnormally short, wide and physically disabled. A phenotype-genotype correlation was noted with missense mutations associated with a milder phenotype than the more severe phenotype seen in individuals with *THRA* truncation mutations. T4 treatment had no effect in any patient (388).

Mutations affecting both TRα1 and α2

A 60-year-old woman presented in childhood with growth failure, macrocephaly, broad face, flattened nasal bridge and a thickened calvarium (389). Her growth improved after T4 treatment during childhood following a radiological opinion suggesting the skeletal features were similar to hypothyroidism. Her 30 and 26 year-old sons presented similarly and were also treated during childhood, resulting in a good growth response. Nevertheless, they each had a persistently abnormal facial appearance and macrocephaly, together with increased BMD between +0.8 and +1.9 SD. In vitro studies demonstrated the mutant TRα1 was a weak dominant negative antagonist but its transcriptional activity could be restored by supra-physiological concentrations of T3, whereas the function of the mutant TRα2 protein did not differ from wild-type (389). Recently, a 27-year-old female with a grossly abnormal skeletal phenotype and low FT4:FT3 ratio was described in association with a N359Y mutation affecting both TRα1 and α2 (390). Her phenotype included intrauterine growth retardation (IUGR) and failure to thrive, macrocephaly, hypertelorism, micrognathia, short and broad nose, clavicular and 12th rib agenesis, elongated thorax, ovoid vertebrae, scoliosis, congenital hip dislocation, short limbs, humeroradial synostosis, and syndactyly (390). This severe and atypical phenotype extends the abnormalities described in patients with *THRA* mutations, and raises questions regarding whether TRα2 has a functional role in skeletal development or whether the RTHα phenotype is more variable and extensive than previously reported.

Together, these reports define a new genetic disorder, RTHα, characterized by profound and consistent developmental abnormalities of the skeleton that recapitulate findings in mice with dominant negative *Thra* mutations (278, 312, 319–321, 391). Initially identified *THRA* mutations resulted in severe phenotypes due to expression of truncated TRα1 proteins with no T3-binding capability but potent dominant-negative activity (382–385). Recognition of individuals with milder mutations and phenotypes has been predicted to be more difficult because disruption of *THRA* does not affect the HPT axis significantly (319).

VII. Thyroid status and skeletal development

A. Consequences of hypothyroidism

Hypothyroidism is the most common congenital endocrine disorder with an incidence of 1 in 1800. Congenital and juvenile acquired hypothyroidism result in delayed skeletal development and bone age with short stature. Abnormal endochondral ossification and epiphyseal dysgenesis are evidenced by "stippled epiphyses" on X-rays. In severe or undiagnosed cases (Figure 8) there is complete postnatal growth arrest and cessation of bone maturation with a complex skeletal dysplasia that includes broad flat nasal bridge, hypertelorism, broad face, patent fontanelles, scoliosis, vertebral immaturity and absence of ossification centers, and congenital hip dislocation (392). Thyroid hormone replacement induces rapid 'catch up' growth and accelerated skeletal maturation, demonstrating the exquisite sensitivity of the developing skeleton to thyroid hormones. Nevertheless, predicted adult height may not be attained and, in such cases, the final height deficit is related to the duration and severity of hypothyroidism prior to diagnosis and treatment (281), although

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the underlying mechanisms remain unknown. Overall, most children with congenital hypothyroidism that are treated early with thyroxine replacement ultimately reach their predicted adult height and achieve normal BMD after 8·5 years follow-up (393, 394).

B. Consequences of thyrotoxicosis

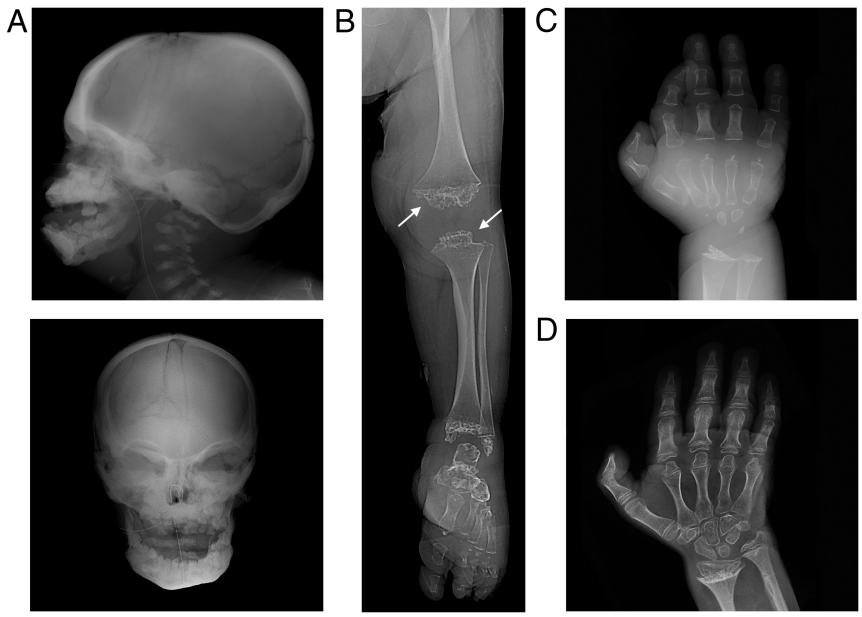
During skeletal development and growth, T3 regulates the pace of chondrocyte differentiation in the epiphyseal growth plates (120, 159, 161, 162, 224). Graves' disease is the commonest cause of thyrotoxicosis in children but remains rare. In contrast to hypothyroidism during childhood, juvenile thyrotoxicosis is characterized by accelerated skeletal development and rapid growth, although advanced bone age results in early cessation of growth and persistent short stature due to premature fusion of the growth plates. In severe cases in young children, early closure of the cranial sutures can result in craniosynostosis (13, 322, 345), while maternal hyperthyroidism per se may also be a risk factor for craniosynostosis (395). These observations demonstrate further the marked sensitivity of developing skeleton to the actions of thyroid hormone.

VIII. Thyroid status and bone maintenance

Numerous studies have investigated the consequences of altered thyroid function on BMD and fracture risk in adults (396–399). Interpretation is difficult as studies are frequently confounded by inclusion of subjects with a variety of thyroid diseases and comparison of cohorts that include combinations of pre- and postmenopausal women or men (400–405). Many studies lack statistical power because of small numbers, cross-sectional design or insufficient follow-up (401, 406–411). Some studies measure TSH but not thyroid hormones, whereas others determine thyroid hormones but not TSH (402, 408). Other problems include inadequate control for confounding factors including: age; prior or family history of fracture; body mass index (BMI); physical activity; use of estrogens, glucocorticoids, bisphosphonates or vitamin D; prior history of thyroid disease or use of thyroxine; and smoking or alcohol intake.

Different methods have also been used for skeletal assessment, including analysis of bone geometry in some studies (411, 412). Regulation of bone turnover has been investigated by histomorphometry in early studies (413–416), measurement of proinflammatory cytokines (417) and a variety of biochemical markers of bone formation [serum alkaline phosphatase, osteocalcin, carboxyterminal propeptide of type 1 collagen (P1NP)] and resorption [urinary pyridinoline and deoxypyridinoline collagen cross-links, hydroxyproline, carboxyterminal cross-linked telopeptide of type 1 collagen (CTX), cathepsin K], which are all elevated in hyperthyroidism and generally correlate with disease severity (401, 408, 418–422). Overall, hyperthyroidism shortens the bone remodeling cycle in favor of increased resorption (Figure 5). This results in a high bone turnover state

Figure 8.



X-rays of a 36 year-old female with severe untreated congenital hypothyroidism

(*X-rays kindly provided by Dr. Jonathan LoPresti, Keck School of Medicine, University of Southern California*)

(A) Lateral and AP skull images showing persistently patent sutures and fontanelles and delayed tooth eruption.

(B) Lower limb X-ray showing severe epiphyseal dysgenesis with grossly delayed formation of secondary ossification centers (arrows).

(C) Hand X-ray demonstrating a 35-year delay in bone maturation (bone age 14 months).

(D) Bone age advanced by 8 years following T4 replacement for a period of only 18 months, demonstrating rapid acceleration of endochondral ossification and "catch up growth".

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with increased bone resorption and formation rates. The frequency of initiation of bone remodeling is also increased. The duration of bone formation and mineralization is reduced to a greater extent than the duration of bone resorption. This leads to reduced bone mineralization, a net 10% loss of bone per remodeling cycle and osteoporosis (415). BMD has also been determined by several methods including dual x-ray absorptiometry (DXA), single-photon absorptiometry (SPA), dual-photon absorptiometry (DPA), quantitative computed tomography (CT) (QCT) (398), ultrasound (400), and high resolution peripheral QCT (HR-pQCT) (423). Although many retrospective and cross-sectional studies of the effects of thyroid dysfunction on bone turnover and mass have been reported, only a small number of studies have been prospective, while even fewer have investigated fracture susceptibility. Interpretation of the literature is, therefore, difficult and complex.

Discriminating thyroid hormone and TSH effects on the skeleton

Studies investigating osteoporosis, fracture and thyroid hormone excess have been conflicting regarding the relative roles of thyroid hormone and TSH (424). Importantly, this issue cannot be resolved in individuals in whom the HPT-axis is intact and the reciprocal relationship between thyroid hormones and TSH is maintained (13). Nevertheless, simultaneously increased thyroid hormone and TSH signaling occurs in three clinical situations: non-autoimmune autosomal dominant hyperthyroidism and RTHβ, as described above, together with Graves' disease, in which TSHR-stimulating antibodies persistently activate the TSHR and increase T4 and T3 production. Conventionally, secondary osteoporosis in Graves' disease is considered to be a consequence of elevated circulating thyroid hormone levels and increased T3 actions in bone. By contrast, TSH has been proposed as a negative regulator of bone remodeling (32) and thus suppressed TSH levels in thyrotoxicosis were suggested to be the primary cause of bone loss. Nevertheless, since Graves' disease is characterized by persistent autoantibody-mediated TSHR stimulation, such patients should be protected. Despite this, Graves' disease remains an established and important cause of secondary osteoporosis and fracture.

The effects of TSH on bone turnover have also been investigated in clinical studies. In patients receiving TSH-suppressive doses of T4 in the management of differentiated thyroid cancer, administration of recombinant human (rh) TSH (rhTSH) increases TSH levels to $>$ 100 mU/L but does not affect circulating T4 and T3 concentrations as patients have previously undergone total thyroidectomy. In this context, treatment of premenopausal women with rhTSH had no effect on serum markers of bone turnover (425–427). In postmenopausal women two studies reported a reduction in bone resorption markers accompanied by an increase in bone formation markers following rhTSH administration (426, 427), while two studies reported no effect (425, 428).

A. Consequences of variation of thyroid status within the reference range

Effect on bone turnover markers

No studies have been reported in premenopausal women. In 3261 postmenopausal women from the Study of Health in Pomerania, higher TSH was associated with increased bone turnover and stiffness but no association was reported with levels of sclerostin. In 2654 men from the same study, however, TSH was not related to bone turnover, stiffness or sclerostin. Importantly, T3 and T4 levels were not determined in this study (429). In a study of 60 postmenopausal women, Zofkova et al reported a correlation between high TSH and low concentrations of the bone resorption marker urinary deoxypyridinoline, but no relationship with the bone formation marker serum procollagen type I C propeptide (PICP) (430), although individuals with treated hypothyroidism, subclinical hyperthyroidism and secondary hyperparathyroidism were included.

Effect on BMD

One study in 2957 euthyroid healthy Taiwanese men and women of varying ages reported a weak inverse correlation between fT4 and BMD but did not identify any relationship between TSH and BMD (431).

A 6-year prospective study of 1278 healthy euthyroid postmenopausal women from 5 European cities (OPUS study) found that thyroid status at the upper end of the normal range was associated with lower BMD (432). This finding has been supported by a number of cross-sectional studies. Thyroid function in the upper normal reference range was associated with reduced BMD in 1426 healthy euthyroid peri-menopausal women (433). A study of 756 euthyroid Korean women aged 65 and older showed BMD was positively correlated with TSH (434). In 581 postmenopausal American women, TSH at the lower end of the reference range was associated with a 5-fold increased risk of osteoporosis compared to TSH at the upper end of the normal range (435). Kim et al (436) showed that low normal TSH levels were associated with lower BMD in 959 Korean postmenopausal women. In 648 healthy postmenopausal women, higher fT4 levels within the normal range were associated with a relationship with deterioration in trabecular bone microarchitecture (437).

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In 1151 euthyroid men and women over the age of 55 years femoral neck BMD correlated positively with TSH and inversely with free T4 (438). The association with free T4 was much stronger than the association with TSH. A population study of 993 postmenopausal women and 968 men from Tromso showed that subjects with TSH below the 2.5th percentile had a low forearm BMD whereas those with TSH above the 97.5th percentile had high femoral neck BMD (439). A study of 677 healthy men aged between 25–45 years found that higher fT3, total T3 and total T4 levels were associated with lower BMD (440).

Fracture risk

Leader et al investigated 13 325 healthy subjects with at least one TSH measurement during 2004 and demonstrated an increased risk of hip fracture during the subsequent 10 years in euthyroid women with TSH levels in the lower normal range, although no effect was found in men (441). Svare et al analyzed prospective data from over 16 000 women and nearly 9000 men in the Hunt2 study and found no relationship between baseline TSH and hip or forearm fracture, but weak positive associations in women between hip fracture risk and both low and high TSH (442). In 130 postmenopausal women with normal thyroid function, Mazzioti et al (443) found that TSH levels at the low end of the normal range were associated with an increased prevalence of vertebral fractures in women previously known to have osteoporosis or osteopenia. Van der Deure et al (438), however, found no relationship between fT4 or TSH and fracture in 1151 euthyroid men and women over the age of 55 years. In the OPUS study thyroid status at the upper end of the normal range was associated with an increased risk of incident nonvertebral fracture, which was increased by 20% and 33% in women with higher fT4 or fT3, whereas higher TSH was protective and the fracture risk was reduced by 35% (432). However, in a 10-year prospective study of a cohort of only 367 women, no associations between fT3, fT4 or TSH and incident vertebral fracture were identified (444).

Overall, thyroid status at the upper end of the euthyroid reference range is associated with lower BMD and an increased risk of fracture in postmenopausal women, although studies cannot discriminate the relative contributions of thyroid hormones and TSH.

B. Consequences of hypothyroidism

The effects of hypothyroidism on bone turnover have been investigated by histomorphometry (413, 416). Manifestations include reduced osteoblast activity, impaired osteoid apposition and a prolonged period of secondary bone mineralization. Consistent with a low bone turnover state, osteoclast activity and bone resorption are also reduced. The effect is a net increase in mineralization without a major change in bone volume, although bone mass may increase as a result of the prolonged remodeling cycle (413, 445). To identify changes in bone mass resulting from hypothyroidism would require long-term follow up of untreated patients, and data from such studies are not available.

Effect on bone turnover markers

No studies have been reported in pre- or postmenopausal women or men.

Effect on BMD

Consistent with histomorphometry data, two studies reported normal BMD in patients newly diagnosed with hypothyroidism (446, 447). A cross-sectional cohort study of 49 patients with well-substituted hypothyroidism was compared to 49 age-and sex-matched controls investigated BMD by DXA, bone geometry by pQCT and bone strength by finite element analysis. The study showed no difference between controls and patients receiving thyroxine (423). A retrospective study of 400 Puerto Rican postmenopausal women also showed no relationship between hypothyroidism and BMD (448). Nevertheless, Paul et al (449) reported a 10% reduction in BMD in the femur but no effect on lumbar spine BMD following T4 replacement in 31 hypothyroid premenopausal women treated for at least 5 years, while Kung et al (450) reported reduced BMD in 26 hypothyroid premenopausal women receiving T4 for between 1–24 years. These studies are difficult to interpret because of the confounding effects of patient compliance to T4 replacement and the likely variability in the adequacy or sustainment of restored euthyroidism during follow-up.

Fracture risk

Although the effects on fracture risk of T4 replacement for hypothyroidism have not been investigated directly, retrospective data failed to identify an association (451–453). Nevertheless, large cross-sectional population studies have identified an association between hypothyroidism and fracture. Patients with a prior history of hypothyroidism or increased TSH concentration had a 2–3 fold increased relative risk of fracture, which persisted for up to 10 years following diagnosis (447, 454–457). One recent study of thyroxine treatment of 74 patients with adult-onset hypopituitarism also suggested the possibility that overtreatment of patients with T4 may increase vertebral fracture risk in some patients, particularly those with coexistent untreated GH deficiency (458).

In postmenopausal women a database cohort study of

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11 155 women over 65 receiving thyroxine for hypothyroidism revealed an increased risk of fracture in patients with a prior history of osteoporosis who were receiving more than 150 μg of T4, suggesting overtreatment is detrimental (459). Nevertheless, Gonzalez-Rodriguez et al did not identify a relationship between hypothyroidism and fracture in Puerto Rican postmenopausal women (448).

Overall, these studies suggest that hypothyroidism per se is unlikely to be related to fracture risk, whereas long-term supraphysiological replacement with thyroid hormones may result in an increased risk of fracture.

C. Consequences of subclinical hypothyroidism

Effect on bone turnover markers

A randomized controlled trial (RCT) in a heterogeneous group of 61 patients with subclinical hypothyroidism demonstrated that treatment with T4 to restore euthyroidism resulted in increased bone turnover at 24 and 48 weeks and reduced BMD after 48 weeks (460).

Effect on BMD and fracture risk

A study of subclinical hypothyroidism in 4936 US men and women aged 65 years and older followed up for 12 years showed no association with BMD or incident hip fracture (461). A prospective study in men aged 65 and older over 4.6 years follow-up also revealed no association between subclinical hypothyroidism and bone loss (462). A prospective cohort study of 3567 community dwelling men over 65 years revealed a 2.3 fold increased risk of hip fracture over 13 years follow-up in men with subclinical hypothyroidism following adjustment for covariates, including use of thyroid hormones (463).

Meta-analysis

Importantly, an individual participant meta-analysis of 70 298 individuals during 762 401 person-years follow up found no association between subclinical hypothyroidism and fracture risk (464).

D. Consequences of subclinical hyperthyroidism

Effect on bone turnover markers

Management of patients with differentiated thyroid cancer frequently involves prolonged treatment with T4 at doses that suppress TSH and may be detrimental to bone. A few studies investigated the effect of TSH suppression on bone turnover in small numbers of patients and findings have been inconsistent (398). Some reported increased bone formation and resorption markers in patients receiving T4 (401, 465), whereas others reported no effect on either resorption or formation markers (409, 466).

Effect on BMD

Most studies in premenopausal women reported no effect of TSH suppression therapy on BMD at any anatomical site, although one study showed BMD may be reduced (467). Early studies reporting the effect of TSH suppression on BMD in postmenopausal women were conflicting as they frequently evaluated TSH only without measurement of thyroid hormones and were thus unable to exclude overt hyperthyroidism. Overall, therefore, these heterogeneous studies should be interpreted with caution. For example, Franklyn et al investigated 26 UK postmenopausal women treated for 8 years and found no effect of TSH suppression on BMD (468), whereas Kung et al studied 46 postmenopausal Asian women and found decreased total body, lumbar spine and femoral neck BMD (469). Direct comparison between these studies is not possible, however, because TSH was fully suppressed in only 80% of patients in one (468), mean calcium intake was low in the other (469), while both were performed in small numbers of patients but from differing ethnic backgrounds. Similar conflicting data have been reported at various anatomical sites in many less well-controlled cross-sectional and longitudinal studies (398, 409, 410). Recent data have similarly been contradictory; a 12-year follow-up study of endogenous subclinical hyperthyroidism in 1317 men and women aged 65 years and older showed no association with BMD (461). Despite this, a 1-year prospective study of 93 women with differentiated thyroid cancer showed that TSH-suppressive therapy resulted in accelerated bone loss in postmenopausal women, but only in the early postoperative period following thyroidectomy (403). In men, no association between subclinical hyperthyroidism and BMD was identified in two recent studies (461, 462), which support overall findings from previous cross-sectional studies, of which only one reported reduced BMD in men receiving TSH-suppressive doses of T4 (470).

Fracture risk

In a retrospective population study of 2004 individuals, subclinical hyperthyroidism was associated with a 1.25-fold increased risk of fracture although no association with TSH concentration was identified and the relationship between fracture and thyroid hormone levels was not reported (471). Nevertheless, a nested case-control study of 213 511 individuals from Ontario reported a 1.9-fold increased fracture risk in patients treated with T4 and demonstrated a clear dose response relationship (472). A prospective cohort study in postmenopausal women with case-cohort sampling identified an association between suppressed TSH and increased fracture risk, with 3–4 fold increases in both hip and vertebral fractures in individuals with TSH suppressed below 0.01 mU/L (473). A 2.5 fold

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increased rate of hospital admission for fracture in subjects with TSH less than 0.05 mU/L (455), and an exponential rise in the association between fracture risk and longer duration of TSH suppression (474) have also been reported. Further analysis demonstrated an increased risk of fracture in patients with hypothyroidism that was strongly related to the cumulative duration of periods with a low TSH due to excessive thyroid hormone replacement (475). Similarly, in the Fracture Intervention Trial, Jamal et al analyzed a subgroup of patients with TSH suppressed below 0.5 mIU/L and found an increased risk of vertebral fracture (476), although individuals with untreated thyrotoxicosis were not formally excluded.

Recently, Lee et al demonstrated in a 14-year prospective follow-up study that men, but not women, with endogenous subclinical hyperthyroidism had a 5-fold increased hazard ratio (HR) of hip fracture, while men with all causes of subclinical hyperthyroidism had a 3-fold increased HR (463). Nevertheless, a prospective study in men aged 65 and older revealed no association between subclinical hyperthyroidism and fracture risk during 4.6 years follow-up, although a weak increased risk of hip fractures in subjects with lower serum TSH was identified (462). No association was seen between hip fracture risk and endogenous subclinical hyperthyroidism in 4936 US men and women aged 65 years and older followed up for 12 years (461).

In summary, large population studies have revealed increased bone turnover, reduced BMD and an increased risk of fracture particularly in postmenopausal women with subclinical hyperthyroidism (447, 455, 456, 473). Importantly, a prospective study of low-risk differentiated thyroid cancer patients treated with suppressive doses of T4 demonstrated an increased risk of postoperative osteoporosis without beneficial effect on tumor recurrence, and the authors suggested that future intervention in the long-term treatment of low risk disease should focus towards avoiding therapeutic harm (477).

Systematic reviews and meta-analyses

Levels of bone resorption and formation markers have been reported to be either elevated or normal in subclinical hyperthyroidism. Similarly, BMD has been found to be either reduced or unaffected, and a comprehensive meta-analysis was inconclusive (478). Heemstra et al analyzed 12 cross-sectional and 4 prospective studies of premenopausal women receiving suppressive doses of T4, but found that a formal meta-analysis could not be performed due to heterogeneity (397). The authors concluded that suppressive doses of T4 are unlikely to affect BMD in premenopausal women. This conclusion supported an earlier review of 8 studies by Quan et al (479). A meta-analysis of 27 studies investigating effects of TSH suppression on BMD identified no effect in premenopausal women or men, but found that suppressive doses of T4 for up to 10 years in postmenopausal women led to reductions in BMD of between 5%–7% (480). Systematic reviews of effects in postmenopausal women receiving suppressive doses of T4 for approximately 8.5 years suggested an increased rate of annual bone loss that results in a reduction of BMD at the lumbar spine of 7% and hip of 5%. These reviews recommended monitoring BMD in postmenopausal women with subclinical hyperthyroidism (397, 398, 479). Nevertheless, although a recent systematic review and meta-analysis of 7 population-based cohorts also suggested that subclinical hyperthyroidism may be associated with an increased risk of hip and nonspine fracture, a firm conclusion could not be reached due to limitations of the cohorts (481).

Importantly, the largest meta-analysis of 70 298 individuals during 762 401 person-years of follow up demonstrated an increased risk of hip and other fractures in individuals with subclinical hyperthyroidism, particularly in those with TSH suppressed below 0.1 mIU/L and those with endogenous disease (464).

E. Consequences of hyperthyroidism

Effect on bone turnover markers and BMD

The effects of thyrotoxicosis on bone turnover are consistent with histomorphometry data. Markers of bone formation and resorption are elevated and correlate with disease severity in pre- and postmenopausal women and men (418–421). In premenopausal women, treatment of thyrotoxicosis resulted in a 4% increase in BMD within one year (482).

Fracture risk

Severe osteoporosis due to uncontrolled thyrotoxicosis is now rare because of prompt diagnosis and treatment, although undiagnosed hyperthyroidism is an important contributor to secondary bone loss and osteoporosis in patients presenting with fracture (483). The presence of thyroid disease as a comorbidity factor has also been suggested to increase 1- and 2-year mortality rates in elderly patients with hip fracture (484). Several studies have investigated the skeleton in untreated thyrotoxicosis or have performed population studies to determine the relationship between hyperthyroidism and fracture. One cross-sectional (456) and four population studies identified an association between fracture and a prior history of thyrotoxicosis. These studies could not determine whether reduced BMD or thyrotoxicosis were causally related to fracture risk, although one prospective study (451) dem-

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onstrated that a prior history of thyroid disease was independently associated with hip fracture following adjustment for BMD. Bauer et al (473, 485) demonstrated that low TSH was associated with a 3–4 fold increased risk of fracture even though a specific relationship between TSH and BMD was not identified. In agreement, Franklyn et al identified an increased standardized mortality ratio due to hip fracture in a follow-up population register study of patients treated with radioiodine for hyperthyroidism (486). One cross-sectional study (487) identified an association between fracture and a prior history of thyrotoxicosis in postmenopausal women. A large population study reported persistence of an increased risk of fracture 5 years after initial diagnosis (456), but others failed to demonstrate a relationship between history of thyrotoxicosis and fracture (452, 488). Recently, however, population studies have shown reduced BMD and an increased risk of fracture in postmenopausal women with thyrotoxicosis (447, 455, 456, 464, 473).

Systematic reviews and meta-analyses

A meta-analysis of 20 studies of patients with thyrotoxicosis calculated that BMD was reduced at the time of diagnosis and there was an increased risk of hip fracture; further investigation of the effect of antithyroid treatment demonstrated the low BMD at diagnosis returned to normal after 5 years (489).

IX. Osteoporosis and genetic variation in thyroid signaling

Associations with BMD

GWAS in osteoporosis cohorts have not identified associations between variation in thyroid-related genes and BMD or fracture (490–492).

Candidate gene studies have investigated relationships between polymorphisms in the *TSHR*, *THRA* and *DIO2* genes and BMD. The *TSHR* D727E polymorphism was associated with serum TSH concentration and with osteoporosis diagnosed by qUS in 150 male subjects compared to 150 controls, whereas the D36H polymorphism was not (493). By contrast, in 156 patients treated for differentiated thyroid cancer the *TSHR* D727E polymorphism was associated with higher BMD at the femoral neck independent of thyroid status, but this association did not persist following adjustment for BMI (494). A similar association between *TSHR* D727E and increased BMD at the femoral neck was identified in 1327 subjects from the Rotterdam study (438).

A candidate gene association study of 862 men over 65 investigated genetic variation in *THRA* and BMD at the femoral neck and lumbar spine (495, 496), while a much larger study investigated the *THRA* locus in relation to BMD, fracture risk and bone geometry in 27 326 individuals from the Genetic Factors for Osteoporosis (GEFOS) consortium and the Rotterdam Study 1 and 2 populations (497). Both studies failed to identify any relationship between variation in bone parameters and genetic variation across the *THRA* locus. In a further study, a cohort of 100 healthy euthyroid postmenopausal women with the highest BMD was selected from the OPUS population, and sequencing of *THRA* revealed no abnormalities, indicating that *THRA* mutations are not a common cause of high BMD (498). Yerges et al, however, identified an association between an intronic SNP in *THRB* and trabecular BMD in the MrOS study (495).

Variation in deiodinases has been described as a potential genetic determinant of bone pathology. Two studies investigated the relationship between deiodinase polymorphisms and skeletal parameters. The *DIO2* T92A polymorphism was associated with decreased femoral neck and total hip BMD and several markers of bone turnover in 154 athyreotic patients treated for differentiated thyroid cancer (499), suggesting a role for *DIO2* in the regulation of bone formation. However, in 641 young healthy men, no relationships between *DIO1* or *DIO2* variants and bone mass were identified (500). Sequencing of *DIO2* in healthy euthyroid postmenopausal women with high BMD from the OPUS population also failed to identify any abnormalities (498).

X. Osteoarthritis and genetic variation in thyroid signaling

Genetics

A role for thyroid hormones in the pathogenesis of OA has been proposed (501). A GWAS of siblings with generalized OA found a nonsynonymous coding variant (rs225014; T92A) that identified *DIO2* as a disease-susceptibility locus (502). An allele sharing approach reinforced evidence of linkage in the region of this locus (503). Replication studies confirmed this by identifying an association between symptomatic OA and a *DIO2* haplotype containing the minor allele of rs225014 and major allele of rs12885300 (504). The relationship between these *DIO2* SNPs and OA, however, was not replicated in the Rotterdam Study population (505), and *DIO2* was not identified in recent meta-analyses (506, 507). Nevertheless, additional data suggested the risk allele of rs225014 may be expressed at higher levels than the protective allele in OA cartilage from heterozygous patients (508), possibly because of epigenetic modifications (509). Increased *DIO2* mRNA and protein expression has also been documented by RT-qPCR and immunohistochemistry in car-

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tilage from joints affected by end-stage OA (508, 510). Furthermore, the rs12885300 *DIO2* polymorphism has been suggested to influence the association between hip shape and OA susceptibility by increasing vulnerability of articular cartilage to abnormal hip morphology (511). Moreover, studies in transgenic rats that overexpress human *DIO2* in chondrocytes indicate these animals had increased susceptibility to OA following provocation surgery (510). Finally, a meta-analysis studying genes that regulate thyroid hormone metabolism and mediate T3 effects on chondrocytes identified *DIO3* as a disease modifying locus in OA (504).

Together, these data suggest that local T3 availability in joint tissues may play a role in articular cartilage renewal and repair, particularly as the associated *DIO2* and *DIO3* polymorphisms do not influence systemic thyroid status (16). Overall, increased T3 availability may be detrimental to joint maintenance and articular cartilage homeostasis.

Mechanism

Adult *Dio2*$^{-/-}$ mice have normal articular cartilage and no other features of spontaneous joint damage, but exhibit increased subchondral bone mineral content (512). In a forced exercise provocation model *Dio2*$^{-/-}$ mice were protected from articular cartilage damage (513). In studies demonstrating that functional DIO2 enzyme is restricted to bone-forming osteoblasts, we showed *Dio2* mRNA expression does not necessarily correlate with enzyme activity (61). An important reason for this discrepancy is that DIO2 protein is labile, undergoing rapid degradation following exposure to increasing concentrations of T4 in a local feedback loop (50, 159). We demonstrated *Dio2* mRNA in growth plate cartilage but could not detect enzyme activity using a high sensitivity assay (61), whereas others showed *Dio2* mRNA expression in rat articular cartilage (510), and increased levels of *DIO2* mRNA (510) and protein (508) in human articular cartilage from joints resected for end-stage OA. In each case, however, enzyme activity was not determined. The significance of increased *DIO2* mRNA in end-stage OA cartilage is therefore uncertain; it is also unknown whether increased *DIO2* expression might represent a secondary response to joint destruction or whether it precedes cartilage damage and might be a causative factor in disease progression.

Transgenic rats overexpressing DIO2 in chondrocytes had increased susceptibility to OA following surgical provocation (510). Nevertheless, a causal relationship between increased *DIO2* expression and OA susceptibility was not established because enzyme activity was not determined (510). This is particularly important because in a previous study, transgenic mice overexpressing *Dio2* in the heart surprisingly displayed only mild thyrotoxic changes in cardiomyocytes (514), likely because the phenotype was mitigated by T4-induced degradation of the overexpressed enzyme (159). Furthermore, siRNA-mediated inhibition of *DIO2* in primary human chondrocytes resulted in decreased expression of liver X receptor α (LXRα) and increased expression of interleukin-1β (IL1β) and IL1β-induced cyclooxygenase-2 (COX2) expression (515). Thus, in contrast to the previous studies, these findings suggest that suppression of *Dio2* results in a proinflammatory response in cartilage. The increase in *DIO2* expression observed in end-stage human OA (508, 510) may, therefore, be a consequence of disease progression resulting from activation of proinflammatory pathways such as the NFκB pathway, which is known to stimulate *Dio2* expression (516, 517).

Although chondrocytes resist terminal differentiation in healthy articular cartilage, a process resembling endochondral ossification occurs during OA in which articular chondrocytes undergo hypertrophic differentiation with accelerated cartilage mineralization (518, 519). ADAMTS5 (520, 521) and MMP13 (522) degrade cartilage matrix and these enzymes are regulated by T3 in the growth plate (205, 222, 223, 523, 524). In articular cartilage, thyroid hormones stimulate terminal chondrocyte differentiation (525) and tissue transglutaminase activity (526). In cocultures of chondrocytes derived from different articular cartilage zones, interactions between chondrocytes from differing zones were identified that modulated responses to T3 and were mediated in part by PTHrP. The authors proposed that communication between zones might regulate postnatal articular cartilage organization and mineralization (527).

Currently, the pathophysiological importance of these studies is difficult to evaluate. Initial genetic studies suggesting that reduced DIO2 activity is associated with increased susceptibility to OA (502) were based on the assumption that the T92A polymorphism results in reduced enzyme activity (528). However, studies showing that transgenic rats overexpressing DIO2 in articular cartilage had increased susceptibility to OA (510) were not consistent with this conclusion, and recent findings of allelic imbalance and increased expression of DIO2 protein in OA cartilage (508, 509) further suggest increased DIO2 activity may be detrimental for articular cartilage maintenance. On the other hand a large GWAS and recent meta-analyses failed to identify *DIO2* as a disease susceptibility locus for OA (505–507), while a recent study in primary human chondrocytes suggests an anti-inflammatory role for DIO2 in cartilage (515). Thus, it remains unclear whether variation in *DIO2* plays a role in osteoarthritis pathogenesis, although the independent identification of *DIO3* as a disease susceptibility locus (504) sup-

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ports a role for control of local tissue T3 availability in the regulation of joint homeostasis.

XI. Summary and future directions

The skeleton is exquisitely sensitive to thyroid hormones, which have profound effects on bone development, linear growth and adult bone maintenance.

Thyroid hormone deficiency in children results in cessation of growth and bone maturation, whereas thyrotoxicosis accelerates these processes. In adults, thyrotoxicosis is an important and established cause of secondary osteoporosis, and an increased risk of fracture has now been demonstrated in subclinical hyperthyroidism. Furthermore, even thyroid status at the upper end of the normal euthyroid reference range is associated with an increased risk of fracture in postmenopausal women.

An extensive series of studies in genetically modified mice has shown that T3 exerts anabolic actions on the developing skeleton, has catabolic effects in adulthood, and these actions are mediated predominantly by TRα1. The importance of the local regulation of T3 availability in bone has been demonstrated in studies that identified a critical role for DIO2 in osteoblasts to optimize bone mineralization and strength. The translational importance and clinical relevance of such studies is highlighted by the characterization of mice harboring deletions or dominant-negative mutations of *Thra*, which accurately predicted the abnormalities seen in patients recently identified with RTHα. Moreover, mice with dominant-negative mutations of *Thra* also represent an important disease model in which to investigate novel therapeutic approaches in these patients. In addition to studies in genetically modified mice, analysis of patients with thyroid disease or inherited disorders of T3 action is consistent with a major physiological role for T3 in the regulation of skeletal development and adult bone maintenance. Analysis of *Tshr*$^{-/-}$ mice and studies of TSH administration in rodents have also suggested that TSH acts as a negative regulator of bone turnover. Nevertheless, the physiological role of TSH in the skeleton remains uncertain, as its proposed actions are not wholly consistent with findings in human diseases and mouse models in which the physiological inverse relationship between thyroid hormones and TSH is dissociated.

Precise determination of the cellular and molecular mechanisms of T3 and TSH actions in the skeleton in vivo will require cell-specific conditional gene targeting approaches in individual bone cell lineages. Combined with genome-wide gene expression analysis, these approaches will determine key target genes and downstream signaling pathways and have the potential to identify new therapeutic targets for skeletal disease.

Recent studies have also identified *DIO2* and *DIO3* as disease susceptibility loci for osteoarthritis, a major degenerative disease of increasing prevalence in the ageing population. These data establish a new field of research and further highlight the fundamental importance of understanding the mechanisms of T3 action in cartilage and bone, and its role in tissue maintenance, response to injury and pathogenesis of degenerative disease.

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