Role of Thyroid Hormone Receptor-α1 in Endochondral Ossification

Graham R. Williams

Molecular Endocrinology Group, Department of Medicine, Imperial College London, London W12 0NN, United Kingdom

Thyroid hormones are important regulators of postnatal skeletal development, linear growth, and bone maintenance. Thyroid hormone deficiency during childhood results in growth retardation and delayed bone age and tooth eruption, with epiphyseal dysgenesis and linear growth arrest evident in severe cases (1). Treatment of hypothyroid children with T_4 results in rapid catch-up of bone age and growth, although short stature may persist in individuals who have prolonged hormone deficiency prior to treatment (2, 3). In contrast, thyrotoxicosis induces premature bone maturation and increases linear growth velocity, although the early closure of the growth plates and premature fusion of the sutures of the skull can lead to persistent short stature and craniosynostosis in children with severe or undiagnosed and continuing hyperthyroidism (4). Overall, these observations reveal exquisite sensitivity of the postnatal skeleton to thyroid hormones and the requirement for euthyroid status to ensure normal bone development and growth.

Endochondral ossification is the mechanism underlying bone formation on a cartilage scaffold and the process by which linear growth proceeds in the epiphyseal growth plates (5). Mesenchyme progenitor cells condense and differentiate into chondrocytes, which proliferate and secrete matrix proteins to form a cartilage template for bone formation. At the primary ossification center a tightly regulated program of chondrocyte organization, proliferation, hypertrophic differentiation, and apoptosis results in cartilage mineralization. Vascular invasion and migration of osteoblasts leads to the replacement of mineralized cartilage with trabecular bone. In addition, mesenchyme progenitors condense within perichondrial regions surrounding cartilage and differentiate into osteoblasts, which, via intramembranous ossification, directly form a collar of cortical bone (5). Subsequently, secondary ossification centers form at the ends of long bones, but they remain separated from the primary ossification center by the epiphyseal growth plates. An organized program of chondrocyte proliferation, hypertrophy, and apoptosis continues in the growth plates. Endochondral ossification thus mediates longitudinal growth until adulthood when the epiphyses fuse and linear growth ceases (5).

All of these developmental processes resulting in the formation of primary and secondary ossification centers, establishment of a mineralized cortical bone collar, and the progression of linear growth are sensitive to thyroid hormones (1, 6, 7). The principal actions of thyroid hormones are mediated by nuclear receptors (TRα1, TRβ1, or TRβ2), which are expressed in tissue-specific patterns and function as T_3-inducible transcription factors (8). Analysis of mouse mutants with disrupted T_3 signaling has revealed that TRα1 is the functionally predominant TR isoform expressed in cartilage and bone (1, 6), and the recent identification of patients with autosomal dominant mutations affecting *THRA* has confirmed the physiological importance and predominance of TRα1 in human skeletal development and postnatal growth (9–12).

Nevertheless, and despite considerable advances in recent years, the cell-specific mechanisms and downstream signaling pathways that mediate the developmental effects of thyroid hormones in cartilage and bone remain incompletely understood. Importantly, the widespread endocrine effects of T_3 have made it difficult to disentangle local cell-specific T_3 actions in cartilage and bone from indirect systemic effects that result from skeletal responses to T_3-inducible cytokines or growth factors produced in other

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Abbreviations: AMI, AF2 mutation inducible; E, embryonic day; micro-CT, microcomputed tomography; P, postnatal day; TR, thyroid hormone receptor.

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organs. In the current issue of *Endocrinology*, Desjardin et al (13) address this difficulty by investigating the role of TRα1 in ossification and postnatal growth using an elegant gene-targeting approach.

$TR\alpha^{AMI}$ mice harbor a floxed *Thra* allele [AF2 mutation inducible (AMI)] and express a mutant $TR\alpha1^{L400R}$ only after Cre-mediated recombination (14). The $TR\alpha1^{L400R}$ mutation disrupts the C-terminal α-helix of TRα1 resulting in expression of a mutant receptor, which has reduced affinity for T_3 binding and is unable to interact with histone acetyl transferase coactivator proteins. As a consequence, $TR\alpha1^{L400R}$ fails to activate T_3 target gene transcription. The mutant receptor has also acquired gain-of-function, dominant-negative activity and interferes with wild-type TRα1, resulting in the loss of T_3 responses in target tissues of heterozygous mutant mice. The dominant-negative activity of $TR\alpha1^{L400R}$ also disrupts TRβ function in vitro, although broad characterization of mice that globally express $TR\alpha1^{L400R}$ suggest the actions of TRβ may surprisingly be preserved in vivo (14, 15). A detailed analysis of the effects of $TR\alpha1^{L400R}$ on TRβ function in the skeleton will be required to resolve this apparent paradox before a complete understanding of the effect of $TR\alpha1^{L400R}$ expression in cartilage and bone in vivo can be obtained. Although Desjardin et al (13) argue that the expression of $TR\alpha1^{L400R}$ in cartilage and bone specifically inhibits T_3 actions mediated by TRα1, they also concede that inhibitory effects on TRβ cannot be excluded at this stage. Nevertheless, Desjardin et al crossed $TR\alpha^{AMI}$ mice with *Col2a1Cre* (16) and *Colα1(I)Cre* (17) mice to generate heterozygous $TR\alpha1^{L400R}$*/C2* and $TR\alpha1^{L400R}$*/C1* mice with the aim of restricting the expression of $TR\alpha1^{L400R}$ to cartilage-forming chondrocytes or bone-forming osteoblasts, respectively.

$TR\alpha1^{L400R}$*/C2* mice express the mutant receptor in chondrocytes in the developing skeleton and growth plate and are euthyroid. Expression of Cre recombinase in *Col2a1Cre* mice commences at embryonic day (E) 12.5, suggesting $TR\alpha1^{L400R}$ will be expressed early in skeletal development in $TR\alpha1^{L400R}$*/C2* mice (13). Detailed analysis of *Col2a1Cre* mice, however, also revealed they express Cre recombinase at significant levels in osteoblasts in primary spongiosa, perichondrium, and periosteum from embryonic day 16.5 (16), suggesting that Cre-mediated recombination occurs in both the chondrogenic and osteoblastic cell lineages, and thus, the expression of $TR\alpha1^{L400R}$ in $TR\alpha1^{L400R}$*/C2* mice may not be completely restricted to chondrocytes.

In keeping with the direct actions of T_3 in chondrocytes, $TR\alpha1^{L400R}$*/C2* mice exhibited delayed bone age and a 20% reduction of linear growth accompanied by characteristic histological features of impaired chondrocyte proliferation and endochondral ossification in the developing secondary ossification centers and growth plates at postnatal days (P) 7, P14, and P21. Morphometric analysis of the shape of the snout and skull base also suggested that the abnormal growth of the skull in $TR\alpha1^{L400R}$*/C2* mice resulted from the impaired ossification of cartilage-derived bones (13). These defects resemble those seen in hypothyroid animals and in mice with global disruption of T_3 signaling (1, 6), indicating that developmental effects of T_3 in the skeleton are mediated primarily by the direct actions of T_3 and TRα1 in chondrocytes. Nevertheless, an important issue to consider is that mutant TRs display dominant-negative activity (18, 19), and additional gain-of-function activities have also been described (20, 21). Thus, the use of $TR\alpha1^{L400R}$ to inhibit T_3/TRα1 signaling does not faithfully reproduce local tissue hypothyroidism and alternative cell-specific approaches will be required to determine the precise physiological role and molecular mechanism of TRα1 action in chondrocytes during endochondral ossification.

At odds with T_3 actions solely in chondrocytes and suggesting involvement of the osteoblast lineage due to expression of Cre recombinase in osteoblasts in *Col2a1Cre* mice (16), microcomputed tomography (micro-CT) analysis of trabecular and cortical bone parameters in femurs from P14, P21, and P35 $TR\alpha1^{L400R}$*/C2* mice revealed decreased bone tissue mineralization density, trabecular bone thickness, and cortical bone area compared with age-matched control mice (13). These findings are difficult to interpret, however, and should be considered cautiously. First, $TR\alpha1^{L400R}$*/C2* mice exhibit profound delay in endochondral ossification and skeletal maturation, and this developmental retardation confounds comparison between age-matched mutant and control mice. Thus, phenotypic differences between groups of mice of the same age may result from secondary effects of developmental delay per se (22) rather than being due specifically to direct effects of $TR\alpha1^{L400R}$ in skeletal cells. Second, there are technical difficulties in the analysis of bones from juvenile mice that arise because of the limits of resolution of micro-CT. The sizes of individual bone trabeculae in mice aged between P14 and P35 are at the limit of reliable detection when micro-CT scanning is performed, even at 5 μm voxel resolution. Indeed, the reported differences in mean trabecular thickness between control and mutant mice of 4 μm ± 1–2 μm represent variances that are less than the magnitude of a single voxel. In this context, therefore, the likely role of TRα1 in cells of the osteoblast lineage during bone formation between P14 and P35 is not possible to define from the trabecular and cortical bone data reported in $TR\alpha1^{L400R}$*/C2* mice.

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Desjardin et al (13) also performed gene expression microarray studies in cartilage using femoral heads obtained from P14 *TRα1*^{L400R}*/C2* mice just prior to the onset of secondary ossification center formation. Unfortunately, these studies did not clearly identify specific TRα1 target genes in cartilage, possibly because of cellular heterogeneity in the tissue samples analyzed or because comparisons were made between age-matched rather than developmentally matched *TRα1*^{L400R}*/C2* and control mice. Nevertheless, cluster analysis did identify changes in the expression of genes known to regulate synthesis and turnover of proteoglycans and extracellular matrix and in genes encoding components of the bone morphogenetic protein, IGF-I, and Wnt signaling pathways that are known to be regulated by T_3 in the skeleton (1, 6). Furthermore, changes in the expression of genes associated with cytoskeleton regulation, the primary cilium, and cell adhesion now identify novel signaling pathways by which T_3 may directly control chondrocyte proliferation and differentiation.

Additionally, Desjardin et al (13) crossed TRα^{AMI} and *Colα1(I)Cre* mice to generate *TRα1*^{L400R}*/C1* mice, which express mutant TRα1^{L400R} in postmitotic bone-forming and mineralizing osteoblasts from E14.5. *TRα1*^{L400R}*/C1* mice did not display an abnormal phenotype at P14 when analyzed by micro-CT at 5 μm voxel resolution and by static histomorphometry. Adults aged 14 weeks were also reported as displaying no phenotype abnormalities when assessed by micro-CT at 8 μm voxel resolution and by dynamic histomorphometry, although the gender of adult mice was not specified, and the minimal data discussed were not presented in the manuscript. Given the technical difficulties with analysis of bone structure from P14 mice using micro-CT and the limited reporting of adults at a single time point, it is unclear at this stage whether expression of TRα1^{L400R} in osteoblasts results in phenotypic abnormalities. If *TRα1*^{L400R}*/C1* mice, like *TRα1*^{L400R}*/C2* mice, do display a phenotype reminiscent of cell-specific disruption of TRα1 action, it is probable the effects of impaired T_3 signaling in osteoblasts would be discrete and manifest in older mice (23). This is because thyroid hormone deficiency results in the prolongation of bone remodeling that leads to slow progressive accumulation of bone mass over an extended period of time. In line with this prediction, we recently showed that adult *TRα1*$^{PV/+}$ mice with germline expression of a dominant-negative TRα1 gradually retain bone with age and develop high bone mass despite severe delays in endochondral ossification and linear growth during development (24, 25). A similar picture is emerging in patients with *THRA* mutations (9–12).

Taken together, the studies reported by Desjardin et al (13) provide important genetic confirmation of current understanding obtained from studies of hypothyroid animals and mice with germline disruptions of T_3 action. Thus, T_3 acts via TRα1 to stimulate endochondral ossification and linear growth. These direct actions of T_3 in chondrocytes involve cross talk with bone morphogenetic protein, IGF-I, and Wnt signaling pathways among others. Furthermore, new regulatory pathways involving cell adhesion, the cytoskeleton, and primary cilium are also implicated by the studies of Desjardin et al, and their relationship to T_3/TRα1 signaling in cartilage merits further investigation in the future.

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Address all correspondence and requests for reprints to: Graham R. Williams, MB BS, PhD, FRCP, Molecular Endocrinology Group, Imperial College London, 10N5 Commonwealth Building, Hammersmith Campus, Du Cane Road, London W12 0NN, United Kingdom. E-mail: graham.williams@imperial.ac.uk.

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