Contrasting Skeletal Phenotypes in Mice with an Identical Mutation Targeted to Thyroid Hormone Receptor α1 or β

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Thyroid hormone (T₃) regulates bone turnover and mineralization in adults and is essential for skeletal development. Surprisingly, we identified a phenotype of skeletal thyrotoxicosis in T₃ receptor βPV (TRβPV) mice in which a targeted frameshift mutation in TRβ results in resistance to thyroid hormone. To characterize mechanisms underlying thyroid hormone action in bone, we analyzed skeletal development in TRα1PV mice in which the same PV mutation was targeted to TRα1. In contrast to TRβPV mice, TRα1PV mutants exhibited skeletal hypothyroidism with delayed endochondral and intramembranous ossification, severe postnatal growth retardation, diminished trabecular bone mineralization, reduced cortical bone deposition, and delayed closure of the skull sutures.

Skeletal hypothyroidism in TRα1PV mutants was accompanied by impaired GH receptor and IGF-I receptor expression and signaling in the growth plate, whereas GH receptor and IGF-I receptor expression and signaling were increased in TRβPV mice. These data indicate that GH receptor and IGF-I receptor are physiological targets for T₃ action in bone in vivo. The divergent phenotypes observed in TRα1PV and TRβPV mice arise because the pituitary gland is a TRβ-responsive tissue, whereas bone is TRα responsive. These studies provide a new understanding of the complex relationship between central and peripheral thyroid status. (Molecular Endocrinology 19: 3045–3059, 2005)
T₃-regulated genes (10). The clinical syndrome of RTH is variable, resulting from both the direct effects of the mutant receptors and the consequences of elevated thyroid hormone concentrations. In addition, the differing ratios of TRα and TRβ proteins that are expressed in individual tissues further complicate the syndrome. In tissues such as the heart, in which TRα predominates, the presence of tachycardia in RTH is likely to be due to the effects of elevated thyroid hormone levels acting via TRα. In contrast, in tissues such as the liver, in which TRβ predominates, the presence of hypercholesterolemia in RTH may result from a combination of local tissue hypothyroidism and the direct dominant-negative actions of mutant TRβ proteins in hepatocytes. Thus, the spectrum of clinical features in RTH is broad and can include reduced weight, cardiac disease, hypercholesterolemia, tachycardia, hearing loss, attention-deficit hyperactivity disorder, decreased IQ, and dyslexia (10–12). This complex spectrum reflects the presence of thyrotoxicosis in some T₃-target tissues but evidence of hypothyroidism in others.

A wide variety of bone phenotypes has been described in RTH, and this is probably because objective studies of the skeleton and growth are available in only a small minority of patients. Features include stippled epiphyses with scattered calcification in the growth plate, high bone turnover osteoporosis and fracture, reduced bone density, craniosynostosis, and various defects of facial bone and vertebral development (7). Growth retardation and short stature have been estimated to occur in 26% of patients with variably delayed bone age in up to 47% (7, 8), although bone age has also been shown to be advanced by more than 2 sds in two families, and lesser degrees of advancement have also been documented (7).

In a previous study, we characterized skeletal development in mutant mice with a PV mutation targeted to the TRβ gene locus (13). The PV mutation was derived from a patient with severe RTH and consists of a C insertion at codon 448, which produces a frameshift of the carboxyl-terminal 14 amino acids of TRβ1. The mutant TRβPV protein cannot bind T₃, fails to transactivate T₃ target genes in vitro, and is a potent dominant-negative antagonist (14). TRβPV mice have very high levels of circulating T₄, T₃, and TSH (14), and we showed they display a phenotype of skeletal thyrotoxicosis (13). We also demonstrated that TRα1 mRNA is expressed at 12-fold higher concentrations than TRβ1 in bone, suggesting that the phenotype of skeletal hyperthyroidism in TRβPV mice results from increased T₃ levels acting via TRα1 (13). To investigate this hypothesis and determine whether TRα1 is functionally predominant in bone, we studied mice carrying the PV mutation targeted to TRα1 (15). The mutant TRα1PV protein also acts as a potent dominant-negative antagonist that interferes with transcriptional activities of the wild-type TRα and TRβ receptors (15). In keeping with other mice with dominant-negative RTH mutations in TRα1 (11, 16), heterozygous TRα1PV/+ mice display only a modest degree of thyroid failure. There were small increases in TSH (1.7-fold) and T₃ (1.15-fold) levels in TRα1PV/+ mice, but no change in circulating T₄ concentrations, and the homozygous mutation was lethal (15). Characterization of bone development in biochemically euthyroid TRα1PV/+ mice, therefore, enabled us to investigate mechanisms of T₃ action in bone and determine whether TRα1 is the major functional TR expressed in the skeleton.

RESULTS

TRα1PV/+ Mice Exhibit Delayed Ossification and Postnatal Growth Retardation

Analysis of bone lengths in TRα1PV/+ mice revealed severe and persistent postnatal linear growth impairment (Fig. 1). No sexually dimorphic influences of the TRα1PV mutation on bone growth or development were observed. TRα1PV/+ tibias were 15–25% shorter than tibias from wild-type littermates at all postnatal ages examined. In contrast, no difference was observed between embryonic d 17.5 (E17.5) and postnatal d 1 (P1) wild-type and TRα1PV/+ mice. TRα1PV/+
ulnas were also markedly shorter than wild-type (12–14% reduction), although the magnitude of growth impairment was less than in the tibia (Fig. 1). This degree of postnatal growth impairment in TRα1PV/+ long bones was much more severe than documented in TRβPV/+ and TRβPV/PV mice (13).

Analysis of skeletal preparations from E17.5 and P1 TRα1PV/+ mice confirmed that bone lengths in mutant mice did not differ from wild type before birth, and the appearance of rib cages and vertebrae from wild-type and TRα1PV/+ mice was similar (Fig. 2A). In these preparations, alizarin red stained ossified bone pink and alcian blue 8GX stained cartilage blue. Nevertheless, analysis of limbs in E17.5 mice revealed a small delay in endochondral ossification in the ulna and radius of the forelimb and in the tibia and fibula of the hindlimb that was evident in all TRα1PV/+ mice examined (reduced alizarin red staining in these regions in TRα1PV/+ mice [n = 7] compared with wild-type [n = 6], arrowed in Fig. 2A). Similar findings were present in neonatal mice (wild-type n = 3; TRα1PV/+ n = 12; data not shown). These observations are in contrast with findings in TRβPV/PV mice, in which E17.5 and P1 skeletons displayed advanced endochondral ossification and were larger than wild-type littermates (13). Examination of the skull in E17.5 and neonatal mice revealed further differences between wild-type and TRα1PV/+ mice. There was no difference in anterior-posterior and biparietal skull dimensions, but the fontanelles in TRα1PV/+ mice were larger and cranial sutures wider than in wild-type littermates [E17.5: 37.9 ± 2.5 vs. 20.8 ± 0.9 (P < 0.001); P1: 9.5 ± 0.8 vs. 4.3 ± 1.2 (P < 0.05), area of open fontanelles and sutures expressed as percentage of total skull area in TRα1PV/+ vs. wild-type mice], indicating delayed fontanelle closure and suture fusion (Fig. 2). Intramembranous bone deposited in frontal and parietal bones of the TRα1PV/+ skull was also more porous and stained less intensely (Fig. 2B). These data demonstrate normal growth dimension, but markedly delayed intramembranous ossification of the skull in TRα1PV/+ mice and contrast with findings in TRβPV/PV mice, in which advanced ossification of the skull with craniosynostosis was demonstrated (13).

Postnatal linear growth in TRα1PV/+ and TRβPV/PV mice was examined in detail (Fig. 3). Tibias from TRα1PV/+ mice were 15%, 17%, 20%, and 16% shorter than wild type at ages 2, 3, 4, and 7 wk, respectively, whereas tibias from TRβPV/PV mice were 11% shorter at 2 wk and only 2% shorter at 4 wk reflecting the accelerated growth spurt between these ages in TRβPV/PV mice (13). Growth impairment in TRα1PV/+ mice was accompanied by reduced ossification of the secondary tibial epiphyses, a finding not seen in TRβPV/PV mice, but which persisted in TRα1PV/+ mice at 3 and 4 wk. Furthermore, hindlimb paws from TRα1PV/+ mice at 3 and 7 wk revealed persistently impaired endochondral bone formation with delayed formation of secondary ossification centers in metatarsal bones at 2 wk and the presence of open metatarsal growth plates at 7 wk (Fig. 3). In contrast, epiphyseal ossification and metacarpal and metatarsal growth plate closure was advanced in 3 wk-old TRβPV/PV mice (13). These data indicate that postnatal linear growth impairment in TRα1PV/+ mice was associated with delayed endochondral ossification, whereas in TRβPV/PV mice it resulted from advanced bone development.

Endochondral ossification in the proximal tibia was analyzed in histological studies in TRα1PV/+ mice (Fig. 4). In wild-type mice the proximal tibia secondary os-

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**Fig. 2.** Skeletal Preparations from Wild-Type (α1PV/+ and TRα1PV/+ Mice Stained with Alizarin Red (Bone) and Alcian Blue 8GX (Cartilage)

A, E17.5 mice, limbs ×10, rib cages ×7.5, and skulls ×11 magnification. Arrows indicate distal fore- and hindlimbs in TRα1PV/+ mice and show reduced alizarin red staining in mutants compared with wild-type littermates. B, Neonatal mice, skulls ×11 and ×30 magnification. Anatomy of the skull sutures, bones, and fontanelles is shown in the adjacent diagram.
sification center was already established with formation of bone trabeculae within the epiphysis at 2 wk. Between 2 and 7 wk there was progressive narrowing of the growth plate with increased epiphyseal trabecular bone deposition as endochondral ossification and bone maturation continued. In contrast, in TRα1/PV/PV mice endochondral ossification was markedly delayed. TRα1/PV/PV tibiae were smaller in all dimensions, and development of the secondary ossification center was initiated at 3 wk, a time at which this process was well advanced in wild-type littermates. Formation of an organized growth plate, and subsequent growth plate narrowing during the progression of endochondral ossification, was also markedly delayed. The histological features in 7-wk TRα1/PV/PV mice were similar to those in 3- to 4-wk wild-type mice, indicating that endochondral ossification was delayed by up to 4 wk in mutants (Fig. 4A). Delayed endochondral ossification in TRα1/PV/PV mice was associated with reduced deposition of calcified trabecular bone, as evidenced by reduced von Kossa staining of undecalcified sections of the tibia in 2-wk-old mutants (n = 3) compared with wild type (n = 3) (Fig. 4B). In particular, mineralization...
of trabecular bone in the secondary epiphysis in wild-type mice was already established by 2 wk of age, whereas in TRα1PV/+ mice, staining in this region was absent. A smaller reduction in von Kossa staining was evident in trabecular bone in the region of the metaphysis in TRα1PV/+ mice. In addition, cortical bone deposition in the tibial diaphysis was reduced by approximately 50% in 2-wk TRα1PV/+ mice compared with wild-type littermates (Fig. 5). These data demonstrate that postnatal growth impairment in TRα1PV/+ mice is associated with a 3- to 4-wk delay in bone formation, reduced trabecular bone mineralization, and impaired cortical bone deposition. The findings contrast with those in TRβPV/PV mice, in which advanced ossification and increased trabecular bone mineralization were evident (13).

To investigate mechanisms underlying delayed ossification in TRα1PV/+ mice, measurements of specific regions in the growth plate were performed (Fig. 6). Histological studies enabled the reserve (RZ), proliferative (PZ), and hypertrophic (HZ) zones of growth plates to be identified (13, 17–19). In situ hybridization was performed to determine the expression of collagen II, a marker of proliferating chondrocytes (20), and allow measurement of growth plate dimensions (Fig. 6A). Between 2 and 4 wk there was progressive narrowing of the growth plate in wild-type mice that was due to the normal proportionate narrowing in each of the RZ, PZ, and HZ regions (Fig. 6B). The growth plate continued to narrow in wild-type mice between 4 and 7 wk, but at a slower rate than in younger animals reflecting growth plate maturation and its imminent quiescence as linear growth tails off toward adulthood (Fig. 6C). In TRα1PV/+ mice, measurement of specific regions of the growth plate were not possible until animals reached 7 wk of age because, before that time, formation of the proximal tibial growth plate was incomplete (Fig. 4). At 7 wk the growth plate in TRα1PV/+ mice was significantly wider than in 7-wk wild-type littermates (P < 0.001) but did not differ in width when compared with growth plates from 4-wk wild-type mice. The finding that the width of the growth plate in 4-wk wild-type mice was similar to the width observed in 7-wk old TRα1PV/+ mice suggested that endochondral ossification was delayed by about 3 wk in TRα1PV/+ mice. Thus, comparisons of individual growth plate zones between 4-wk wild-type and 7-wk TRα1PV/+ mice were made to investigate why ossification was delayed in TRα1PV/+ mutants. These comparisons revealed that the PZ and HZ regions in TRα1PV/+ mice were narrower than in wild-type (5% and 10%, respectively; P < 0.05) but the RZ width was similar to wild type (Fig. 6B). Taken together, these data confirm that endochondral ossification in TRα1PV/+ mice is delayed by approximately 3–4 wk and suggest this delay is due to impaired transition of immature RZ chondrocytes into the PZ, resulting in proportionally reduced numbers or dimensions of proliferating and hypertrophic chondrocytes. The data contrast with findings in TRβPV/PV mice, in which disproportionate and accelerated narrowing of the PZ and HZ regions accounted for premature growth plate quiescence by 4 wk of age (13).

**TRα1PV/+ Mice Exhibit Skeletal Hypothyroidism**

We previously identified that fibroblast growth factor receptor-1 (FGFR1) is a T3-target gene in bone. Skeletal FGFR1 expression was reduced in TRα-null (TRα0/0) mice, which display a hypothyroid skeletal phenotype (19), but was increased in TRβPV/PV mice (13). In contrast, comparison of 3-wk-old wild-type mice with 7-wk-old TRα1PV/+ mice (equivalent ages of growth plate maturation, Figs. 4 and 6) revealed that FGFR1 mRNA expression in both chondrocytes and osteoblasts was markedly reduced in TRα1PV/+ mice (Fig. 7). These data demonstrate that TRα1PV/+ mice display severe skeletal hypothyroidism.

**GH and IGF-I Signaling in the Growth Plate Is Impaired in TRα1PV/+ Mice, But Increased in TRβPV Mice**

In view of the impaired transition of chondrocytes from RZ to PZ in TRα1PV/+ mice, we investigated further by examining GH/IGF-I signaling in the growth plate. The GH/IGF-I pathway is initiated by GH, which activates the GH receptor (GHR) in growth plate chondrocytes. GH either acts directly on growth plate chondrocytes to regulate their proliferation and differentiation or stimulates local production of IGF-I, which subsequently acts in a paracrine manner to stimulate the IGF-I receptor (IGF-IR). IGF-I also exerts GH-independent actions on growth plate chondrocytes (21). GH stimulation results in activation of a signaling cascade that involves signal transducer and activator of transcription (STAT)5 (22, 23), whereas stimulation of
IGF-IR results in activation of protein kinase B/Akt signaling (24, 25).

The GH/IGF-I pathway was investigated in growth plates from wild-type, TR\textsuperscript{1PV/H11001}, TR\textsuperscript{PV/H11001}, and TR\textsuperscript{PV/PV} mice by in situ hybridization and immunohistochemistry. In 4-wk wild-type mice, GHR was expressed at low levels only in prehypertrophic chondrocytes at the junction between the PZ and HZ. GHR expression was markedly increased in TR\textsuperscript{PV/PV} mice and extended throughout the PZ, whereas increased expression was also observed in TR\textsuperscript{PV/H11001} heterozygotes, but this was restricted to prehypertrophic chondrocytes. In contrast, GHR expression was absent from the growth plate in TR\textsuperscript{1PV/H11001} mice, although low levels of expression were evident in immature chondrocytes populating the incompletely formed growth plate from the region of the developing secondary epiphysis (Fig. 8). Low levels of IGF-I expression were also observed in these immature chondrocytes in TR\textsuperscript{1PV/H11001} mice, but not in the growth plate itself. In contrast, there were no differences in levels of IGF-I expression in TR\textsuperscript{PV/PV} or TR\textsuperscript{PV/PV} mice compared with wild type, in which IGF-I mRNA was expressed in proliferating chondrocytes (Fig. 8). The patterns of expression of the IGF-IR were similar to those observed for expression of GHR. In wild-type animals IGF-IR was restricted to prehypertrophic chondrocytes. Expression was increased in the same region in TR\textsuperscript{PV/PV} heterozygotes but was markedly increased throughout the growth plate in TR\textsuperscript{PV/PV} mice. In the TR\textsuperscript{1PV/PV}, IGF-IR expression was not detected in the growth plate but was present in immature chondrocytes located in the secondary epiphysis (Fig. 8).

To investigate whether the absence of GHR, IGF-I, and IGF-IR expression from growth plates in TR\textsuperscript{1PV/H11001} mice was because of the immaturity of the TR\textsuperscript{1PV/PV} growth plate, we compared levels of expression in 2-, 3-, and 4-wk-old wild-type, TR\textsuperscript{1PV/PV}, TR\textsuperscript{PV/PV}, and TR\textsuperscript{PV/PV} mice (Fig. 9 and data not shown). Expression of all three mRNAs was absent from growth plates of TR\textsuperscript{1PV/PV} mice at all ages but was present at low levels in immature chondrocytes in the region of the secondary epiphysis, consistent with findings in Fig. 8.
In wild-type mice, levels of GHR and IGF-IR decreased with age between 2 and 4 wk and became localized to prehypertrophic chondrocytes (Figs. 8 and 9 and data not shown). In contrast, in TR\textsuperscript{\alpha1PV+/} and TR\textsuperscript{\betaPV/PV} mice, expression of both GHR and IGF-IR remained persistently increased throughout the growth plate at all ages (Figs. 8 and 9 and data not shown). No changes in expression of IGF-I mRNA were observed in wild-type or mutant mice. These data indicate that altered patterns of expression of GHR and IGF-IR mRNAs in TR\textsuperscript{\alpha1PV+/}, TR\textsuperscript{\betaPV/+}, Heterozygote, and TR\textsuperscript{\betaPV/PV} Mice are not related to the maturity of the growth plate per se and suggest they result from altered skeletal T3 signaling as a consequence of the TR\textsuperscript{\alpha1PV+} or TR\textsuperscript{\betaPV} mutation.

To investigate whether changes in mRNA expression correlated with changes in functional activation of GHR and IGF-IR, we investigated the STAT5 and Akt downstream signaling pathways by immunohistochemistry. In TR\textsuperscript{\alpha1PV+/} mice, basal expression of STAT5 and Akt was no different than that of wild type, whereas concentrations of phosphorylated STAT5 and phosphorylated Akt were markedly reduced (Fig. 10). Thus, reduced levels of GHR and IGF-IR mRNAs in TR\textsuperscript{\alpha1PV+/}, TR\textsuperscript{\betaPV/+}, and TR\textsuperscript{\betaPV/PV} mice are not related to the maturity of the growth plate and suggest they result from altered skeletal T3 signaling as a consequence of the TR\textsuperscript{\alpha1PV} or TR\textsuperscript{\betaPV} mutation.

In wild-type mice, levels of GHR and IGF-IR decreased with age between 2 and 4 wk and became localized to prehypertrophic chondrocytes (Figs. 8 and 9 and data not shown). In contrast, in TR\textsuperscript{\betaPV/+} and TR\textsuperscript{\betaPV/PV} mice, expression of both GHR and IGF-IR remained persistently increased throughout the growth plate at all ages (Figs. 8 and 9 and data not shown). No changes in expression of IGF-I mRNA were observed in wild-type or mutant mice. These data indicate that altered patterns of expression of GHR and IGF-IR mRNAs in TR\textsuperscript{\alpha1PV+/}, TR\textsuperscript{\betaPV/+}, and TR\textsuperscript{\betaPV/PV} mice are not related to the maturity of the growth plate per se and suggest they result from altered skeletal T3 signaling as a consequence of the TR\textsuperscript{\alpha1PV} or TR\textsuperscript{\betaPV} mutation.

DISCUSSION

We have demonstrated that TR\textsuperscript{\alpha1PV+} mice exhibit a severe 25% reduction in postnatal linear growth, a 3- to 4-wk delay in endochondral ossification, diminished trabecular bone mineralization, reduced cortical bone deposition, and delayed intramembranous ossification. Reduced expression of the T3-target gene FGFR1 indicates that skeletal hypothyroidism is responsible for this phenotype. The findings contrast with TR\textsuperscript{\betaPV} mice, in which skeletal thyrotoxicosis was documented by increased FGFR1 expression, accelerated
early linear growth, increased trabecular bone mineralization, and advanced endochondral and intramembranous ossification that resulted in short stature and craniosynostosis (13). We previously identified that FGFR1 is a T3-target gene in bone (19), and the major role of FGFR1 in skeletal development is to regulate intramembranous ossification of the skull (26). Activating mutations of FGFR1 cause Pfeiffer’s craniosynostosis syndrome (27), whereas craniosynostosis also occurs in severe childhood thyrotoxicosis (9). The presence of delayed closure of the skull sutures in TRβ/H9251/H11001 mice, together with craniosynostosis in TRβ/H9252/PV/PV mice, suggests FGFR1 mediates important T3 effects that regulate intramembranous ossification. Nevertheless, prominent phenotypes in TRβ/H9251/H11001 and TRβ/H9252/PV mice involve abnormalities of endochondral bone formation. Thus, we investigated GH and IGF-I signaling in the growth plate, because they are major regulators of endochondral ossification and growth (21, 28, 29). The effects of GH were originally proposed to be mediated by liver-derived IGF-I (30), but this was challenged when IGF-I expression was identified in many tissues (21). A “dual effector theory” for GH action was proposed (31) and extrapolated to the growth plate (32). In this model GH initiates differentiation of PZ chondrocytes directly and is proposed to induce local IGF-I production in proliferating chondrocytes. Local IGF-I then acts in an autocrine/paracrine manner to stimulate clonal expansion and chondrocyte proliferation, resulting in longitudinal growth. However, evidence from IGF-I knockout (IGF-I−/−), liver-specific IGF-I knockout (LID), acid-labile subunit knockout (ALSKO) (acid-labile subunit forms a ternary complex with IGF-I and IGF-I binding protein-3 to stabilize serum IGF-I and facilitate its endocrine actions), and LID+ALSKO double-knockout mice have demonstrated that a threshold concentration of circulating IGF-I is also necessary for bone growth. Nevertheless, tissue IGF-I also plays an essential role because IGF-I−/− mice are much more growth retarded than LID+ALSKO double-knockout mice (33–37). Data from GHR knockout (GHR−/−), IGF-I−/−, and GHR−/−IGF-I−/− double mutants indicate that GH and IGF-I act on the growth plate by both independent and overlapping pathways, with IGF-I being the major determinant of embryonic and postnatal growth, and its actions being modulated by GH in the postnatal period (29). It has further been suggested that, because only 17% of somatic growth can be attributed to processes that do not require an intact GH/IGF-I axis, GH and IGF-I pathways in the growth plate act as a point of convergence and participate in the actions of most growth-promoting molecules (29).

This concept is supported by studies showing that IGF-I is stimulated by T3 in osteoblastic cells (38, 39) and IGF-IR is T3 responsive in chondrocyte cultures (40). A recent study also showed that T3 treatment of hypophysectomized rats resulted in increased GHR expression in the growth plate (41), although a previous study showed that GHR expression in the growth plate was independent of thyroid status (42). In addi-

![Fig. 9. In Situ Hybridizations (×200 Magnification) for IGF-IR Expression in Tibial Growth Plates from 2-, 3-, and 4-wk Wild-Type (TRβ+/+), Heterozygote TRβPV+/, and TRβPV/PV Mice](image-url)
tion to effects on local growth plate GH/IGF-I signaling, T₄ and T₃ influence pituitary GH secretion (43).

Abnormalities of the GH/IGF-I axis have been documented in various TR knockout mice: TRα₁/-/- mice (which lack TRα1 and TRβ) have GH- and mild IGF-I deficiency (44), and GH replacement restores their growth but does not improve defective ossification (45); TRα₀/o₀ mice have normal GH production (17); TRβ/-/- mice have mildly reduced GH production (46); and TRα₂/-/- mice (which lack TRα2 but overexpress TRα1) have normal GH levels but are IGF-I deficient (47). We previously showed that TRα₁PV/- mice have normal pituitary GH production (15), whereas GH expression is reduced by 80% and circulating IGF-I is reduced by 40% in TRβPV/PV mutants (14, 48). These data from various TR mutant mice indicate that T₃, acting mainly via TRβ, regulates systemic GH/IGF-I signaling pathways in vivo. Nevertheless, the presence of delayed endochondral ossification in TRα₁PV mice despite normal levels of GH, and the presence of accelerated ossification in TRβPV/PV mice in the face of low levels of GH and IGF-I, is discordant with the known actions of GH/IGF-I in the growth plate. These findings strongly suggest that the skeletal consequences of the PV mutation result from dysregulated local GH/IGF-I signaling in the growth plate.

Data in Figs. 8–11 support this by clearly showing that GHR and IGF-IR expression and signaling are reduced in TRα₁PV mice (skeletal hypothyroidism) but increased in TRβPV/PV mice (skeletal thyrotoxicosis). Nevertheless, the increase in GHR expression in TRβPV/PV growth plates (Fig. 8) was accompanied by a disproportionately small rise in activated STAT5 (Fig. 11). This finding reflects the impaired GH production observed in these mice (14) and demonstrates that the net effect of the TRβPV mutation results from systemic and local consequences on GH action. In contrast, IGF-I expression was unchanged in TRβPV/PV and TRβPV/PV mice compared with wild type and was undetectable in TRα₁PV growth plates, suggesting that TRα and/or GHR activity are necessary for IGF-I expression but indicating that growth plate IGF-I expression is not responsive to increased T₃ or GH action.

Fig. 10. Immunohistochemistry (×200 Magnification) of STAT5 (Upper Panels), pSTAT5 (Second Row), Akt (Third Row) and pAkt (Fourth Row) ProteinExpression in Tibial Growth Plates from Wild-Type (TRα₁+/+) and TRα₁PV/+ Mice

The right-hand column labeled “Controls” shows parallel experiments in which primary antibody was omitted from the immunohistochemistry protocol and which show that staining of STAT and Akt proteins occurred only in the presence specific primary antibody. pSTAT5, Phosphorylated STAT5; pAkt, phosphorylated AKT.
Nevertheless, increased activation of Akt was observed in TRβPV/PV mice and was independent of changes in IGF-I expression, instead correlating with increased IGF-IR expression. These findings suggest that levels of IGF-IR, rather than IGF-I ligand, are limiting in the growth plate. An alternative possibility is that an unidentified T3-stimulated, IGF-I independent pathway could increase Akt activation in TRβPV/PV mice. Taken together, these data indicate that local GH/IGF-I actions mediate important effects of T3 on endochondral ossification.

Nevertheless, in TRα1PV/− mice with skeletal hypothyroidism and reduced GHR and IGF-IR activity, growth plates were observed to be wider than in wild-type mice (Fig. 4), whereas in TRβPV/PV mice, with skeletal thyrotoxicosis and increased GH and IGF-IR signaling, growth plates were narrower (13). In contrast, growth plates were observed to be narrower in GHR−/− and IGF-I−/− mice compared with wild type (29, 36, 49), suggesting that T3 exerts important effects on linear growth that are independent of GH and IGF-I. Indeed, TRα and TRβ are expressed in growth plate chondrocytes (50–53). T3 inhibits clonal expansion and proliferation but promotes hypertrophic differentiation of primary chondrocytes in suspension culture (53), and additional studies have shown T3 regulates the spatial organization of chondrocyte columns and is required for terminal hypertrophic differentiation (54). In contrast, IGF-I stimulates chondrocyte proliferation and differentiation (28). Furthermore, growth retardation in hypothyroidism results from disrupted growth plate architecture, impaired vascular invasion of the growth plate, and inhibition of hypertrophic chondrocyte differentiation (18, 55). Again, differences are apparent as growth retardation in GH and IGF-I deficiency results from a combination of impaired chondrocyte proliferation and a reduction in the linear dimension of terminal hypertrophic chondro-

Table 1. Changes in GH/IGF-I Signaling in TRα1PV and TRβPV Mice

<table>
<thead>
<tr>
<th>Pituitary GH Production</th>
<th>Growth Plate</th>
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<tr>
<td></td>
<td>GHR mRNA</td>
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<tr>
<td>α1PV</td>
<td>Normal</td>
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pSTAT5, Phosphorylated STAT5; pAkt, phosphorylated Akt.
cytes (28, 29, 36, 49, 54). Together, these considerations indicate that regulation of growth and endochondral ossification by T3 involves both GH/IGF-I-independent and GH/IGF-I-dependent pathways.

In these studies, we showed that $\text{TR}_{\alpha}^{-1}^{\text{PV}}$ mice display skeletal hypothyroidism despite the presence of biochemical euthyroidism. In contrast, $\text{TR}_{\beta}^{\text{PV}}$ mice have severe RTH but a phenotype of skeletal thyrotoxicosis (13). This paradox results from differing effects of the PV mutations at the level of the hypothalamic-pituitary-thyroid axis and in bone (Fig. 12). The hypothalamus and pituitary predominantly express $\text{TR}_{\beta}$, and mutation or deletion of $\text{TR}_{\beta}$ results in impaired feedback regulation of TSH and the syndrome of RTH with thyrotoxic levels of T4 and T3 and elevated TSH concentrations (14, 44, 46, 56–58). In this situation, the pituitary displays tissue hypothyroidism. In contrast, mutation or deletion of $\text{TR}_{\alpha}$ does not interfere significantly with feedback regulation of TSH, and minor changes in circulating T4 and T3 levels result from impaired hormone production in the thyroid gland (11, 15–17, 58, 59). In this situation the pituitary functions normally and systemic T4 and T3 levels lie within or close to the normal range. Together with data from other mutant mice (reviewed in Refs. 12, 60, and 61), these considerations establish that $\text{TR}_{\alpha}$ is the physiological mediator of negative feedback control of TSH secretion. In contrast, our previous studies suggest that $\text{TR}_{\alpha}$ is the major functional TR in bone (13, 17, 19). In the current studies, demonstration of skeletal hypothyroidism and impaired ossification in $\text{TR}_{\alpha}^{-1}^{\text{IV}}$ mice establishes that $\text{TR}_{\alpha}$ acts directly in bone as a physiological regulator of skeletal development. In this context, it is apparent that the skeletal consequences of disrupted $\text{TR}_{\beta}$ function in $\text{TR}_{\beta}^{\text{PV}}$ mice result from impaired inhibition of TSH and the resulting elevated T4 and T3 concentrations, which act via $\text{TR}_{\alpha}$ in bone to induce skeletal thyrotoxicosis (13, 62). In contrast, the skeletal hypothyroidism in $\text{TR}_{\alpha}^{-1}^{\text{IV}}$ mice results from locally impaired $\text{TR}_{\alpha}$ function in bone.

Our analysis of $\text{TR}_{\alpha}^{-1}^{\text{IV}}$ and $\text{TR}_{\beta}^{\text{PV}}$ mice has provided a new understanding of the complex relationship between central pituitary thyroid status and peripheral skeletal thyroid status that arises because the pituitary gland is a $\text{TR}_{\beta}$ target tissue, whereas bone is a $\text{TR}_{\alpha}$ target organ. The model in Fig. 12 can be extended to understand the relationship between central and peripheral thyroid status in any T3-target tissue, depending on whether the peripheral tissue in question is $\text{TR}_{\alpha}$ or $\text{TR}_{\beta}$ responsive. Thus, in the heart, a $\text{TR}_{\beta}$-responsive organ, features of thyrotoxicosis are seen in mice with $\text{TR}_{\beta}$ mutation (63), whereas features of hypothyroidism are seen in $\text{TR}_{\alpha}$ mutants (59). In contrast, in the liver, a $\text{TR}_{\alpha}$ target tissue, a hypothyroid phenotype of impaired cholesterol clearance is seen in $\text{TR}_{\alpha}$ mutants but not in $\text{TR}_{\alpha}$ mutants (64).

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**Fig. 12.** Relationship between Pituitary and Skeletal Thyroid Status Revealed by Analysis of $\text{TR}_{\alpha}^{-1}^{\text{IV}}$ and $\text{TR}_{\beta}^{\text{PV/PV}}$ Mice

Bone is a $\text{TR}_{\alpha}$-responsive tissue, whereas pituitary is $\text{TR}_{\beta}$ responsive. The $\text{TR}_{\alpha}^{-1}^{\text{IV}}$ mutation does not affect pituitary T3 responses because mutant $\text{TR}_{\alpha}^{-1}^{\text{IV}}$ concentrations are too low to interfere with $\text{TR}_{\beta}$. In bone, however, T3 responses are severely impaired because of high concentrations of dominant-negative $\text{TR}_{\alpha}^{-1}^{\text{IV}}$, resulting in skeletal hypothyroidism. In contrast, the $\text{TR}_{\beta}^{\text{PV}}$ mutation disrupts pituitary T3 responses and causes RTH, resulting in elevated circulating thyroid hormone concentrations and reduced GH production. In bone, mutant $\text{TR}_{\beta}^{\text{PV}}$ concentrations are too low to interfere with $\text{TR}_{\alpha}1$, and elevated T4 and T3 concentrations hyperstimulate $\text{TR}_{\alpha}$, resulting in skeletal thyrotoxicosis. TRE, Thyroid response element.
MATERIALS AND METHODS

TRα1PV and TRβ1PV Mutant Mice

Animal studies were conducted in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the National Cancer Institute Animal Care and Use Committee. Wild-type, heterozygous (TRα1PV/+ and TRβ1PV/+) and homozygous (TRβ1PV/PV) mutant mice were bred and genotyped as described elsewhere (14, 15). Both TRα1PV and TRβ1PV strains were generated from genomic clones isolated from a 129Sv mouse genomic library and transfected into TC-1 embryonic stem cells. Both mutant strains have a mixed C57BL/6J and NIH Black Swiss genetic background. Initial studies have revealed that TRα1PV mice exhibit growth retardation with only minor alterations in circulating thyroid hormones (15). In contrast, gross RTH is present in homozygous TRβ1PV/PV mice, with milder thyroid dysfunction in heterozygous TRβ1PV/+ mutants (14). Detailed analysis of bone development in TRβ1PV/PV mice has revealed accelerated growth, advanced bone age, and short stature resulting from skeletal thyrotoxicosis (13).

Skeletal Preparations

E17.5, P1, P14, P21, P28, and P49 male and female littermate mice were obtained. E17.5 and neonatal mice and limbs from P14, P21, P28, and P49 animals were fixed in 95% ethanol before staining with alizarin red and alizarin blue GX as described previously (13). Skeletal preparations were photographed using a Leica MZ 75 binocular microscope (Leica AG, Heerbrugg, Switzerland), Leica KL 1500 LCD light source, Leica DFC 320 digital camera, Leica IM50 Digital Image Manager, and Leica TWIN Module DFC 320 image acquisition software. Bone lengths from wild-type and TRα1PV/+ male and female littersmates were determined digitally after linear calibration of pixel size using the image acquisition software. Skull dimensions and open fontanelle and suture areas were calculated using Image J v1.33u software (http://rsb.info.nih.gov/ij/). The assessment of ossification stage in E17.5 and neonatal mice, as determined by the amount of alizarin red staining relative to alizarin blue, was more subjective (Fig. 2). In these studies differences that were observed in all mutant mice examined compared with wild-type littersmates were considered to be indicative of a difference in the degree of ossification.

Histology

Limbs were fixed for 48–72 h in 10% neutral buffered formalin followed by decalcification in 10% formic acid and 10% neutral buffered formalin at 20 C. E17.5 and P1 limbs were decalcified for 5 d and P49 limbs were decalcified for 7 d. Paraffin-embedded 3-μm sections were taken from anatomically oriented bones (three to five parallel levels per bone depending on the age of the animal; 20 sections per level) and stained with hematoxylin and eosin (Pioneer Research Chemicals, Colchester, UK) or van Gieson and alcan blue BGX, as described elsewhere (13, 18). Some limbs from E17.5 and P14 mice were also fixed for 48–72 h in 10% neutral buffered formalin and frozen in paraffin without prior decalcification for determination of mineralization by von Kossa staining of 3-μm cryosections with neutral red counterstain (19).

In Situ Hybridization and Analysis of Growth Plate Dimensions

mRNA expression was analyzed in growth plate sections from P14, P21, P28, and P49 mice using collagen II, collagen X, FGF1, IGF-I, IGF-IR, and GHR cRNA probes. A bacterial neomycin resistance gene cRNA probe (Boehringer Mannheim, Lewes, Sussex, UK) was used as a negative control for all hybridizations, and collagen II (GenBank accession no. 2982–3689; GenBank accession no. L48440) and X (GenBank accession no. 418–858; GenBank accession no. AJ31848) probes were used to identify proliferative and hypertrophic zones in growth plate sections, as described in previous studies in which we optimized in situ hybridization methods (13, 18, 19). A rat FGF1 (nucleotides 104–603; GenBank accession no. S54008) partial cDNA was isolated by RT-PCR as described previously (18, 19) from osteoblastic ROS 17/2.8 cells (65). The rat IGF-I partial cDNA (GenBank accession no. 61–314; GenBank accession no. D00698) was a gift from Dr. Cécile Kedzia (Institut National de la Santé et de la Recherche Médicale, Paris, France). Mouse IGF-I (nucleotides 1063–1690; GenBank accession no. XM_133508) and GHR (nucleotides 470–711; GenBank accession no. NM_010284) partial cDNAs were isolated by RT-PCR as described elsewhere (18, 19) from chondrogenic ATDC5 cells (66) with the following primers: IGF-1R, forward 5′-GAAGACCCACCATCAAAAT-3′, reverse 5′-GAAGAGGACAGGGACCAAGC-3′; GHR, forward 5′-GACCCCCAGGACATTCACGAG-3′, reverse 5′-CAGGTTGCACATTTCCGTCAAC-3′. PCR products were subcloned into pGEM-T (Promega, Southampton, Hampshire, UK) and sequenced. FGF1, IGF-I, IGF-IR, and GHR constructs were linearized with SpeI, BamHI, Dral, and SpeI, and digoxigenin-labeled cRNA probes were synthesized using T7, T3, T7, and T7 RNA polymerases, respectively (Boehringer Mannheim). In situ hybridizations using alkaline phosphatase-labeled probes were performed on 3-μm deparaffinized sections as described elsewhere (13, 18, 19). Studies were performed on at least three mice per genotype in duplicate, and repeat experiments were performed on three separate occasions. Measurements at four separate positions across the width of growth plates were obtained, using a Leica DM LB2 microscope, Leica DFC 320 digital camera, Leica IM50 Digital Image Manager, and Leica TWIN Module DFC 320 image acquisition software, to calculate mean values for the heights of the RZ, PZ, HZ, and total growth plate in sections from wild-type and TRα1PV/+ mice. Results from adjacent levels of sectioning were compared to ensure consistency of the data. Cortical bone width measurements were performed at four separate positions in the midshaft of the tibia and adjacent levels of sectioning were compared. All studies are performed with the observer blinded to the genotype. For histology and histomorphometry analyses, at least three animals per genotype were examined.

Immunohistochemistry

Activation of IGF-IR and GHR downstream signaling was examined by immunohistochemical analysis of protein kinase B (Akt) and signal transducer and activator of transcription-5 (STAT5) expression in wild-type, TRα1PV/+ and TRβ1PV/PV growth plates. Sections were deparaffinized and rehydrated in ethanol and PBS. Sodium citrate antigen retrieval was performed for 6 min in a microwave oven on medium setting. Endogenous peroxidase activity was blocked with 1% H2O2 in methanol for 15 min at room temperature. Sections were then blocked with 5% fetal calf serum (Sigma Chemical Co., St. Louis, MO) in PBS with 0.5% Tween 20 for 1 h at room temperature, before addition of primary antibody and incubation overnight at 4 C. Polyclonal antibodies used to detect expression of Akt (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphorylated Akt (Cell Signaling Technology, Inc., Beverly, MA), STAT5 (Santa Cruz), and phosphorylated STAT5 (Santa Cruz) were diluted 1:175, 1:50, 1:200, and 1:140, respectively. Sections were subsequently incubated with peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Inc., Hercules, CA) diluted 1:2000 to 1:1800 for 30 min at room temperature. Peroxidase activity was detected using 3,3'-diaminobenzidine.
dine containing 0.02% H$_2$O$_2$ (Sigma). Negative controls lacking primary antibody were performed in parallel in all experiments, as described elsewhere (53, 67). Studies were performed on at least three mice per genotype in duplicate, and repeat experiments were performed on three separate occasions.

**Statistical Analysis**

Data were expressed as mean ± SEM. Differences between groups were examined for statistical significance using Student’s t test, in which P values <0.05 were considered significant.

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