Advanced Bone Formation in Mice with a Dominant-negative Mutation in the Thyroid Hormone Receptor β Gene due to Activation of Wnt/β-Catenin Protein Signaling

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Background: Thyroid hormone (T₃) regulates skeletal development by unknown mechanisms.

Results: Canonical Wnt signaling was inhibited by T₃ in osteoblastic cells, but activated in ThrbPV/PV mutant mice that display advanced bone formation.

Conclusion: The mutant thyroid hormone receptor (TRβPV) activates skeletal Wnt signaling in vivo.

Significance: T₃ and Wnt signaling pathways interact during bone development.

Thyroid hormone (T₃) acts in chondrocytes and bone-forming osteoblasts to control bone development and maintenance, but the signaling pathways mediating these effects are poorly understood. ThrbPV/PV mice have a severely impaired pituitary-thyroid axis and elevated thyroid hormone levels due to a dominant-negative mutant T₃ receptor (TRβPV) that cannot bind T₃ and interferes with the actions of wild-type TR. ThrbPV/PV mice have accelerated skeletal development due to unknown mechanisms. We performed microarray studies in primary osteoblasts from wild-type mice and ThrbPV/PV mice. Activation of the canonical Wnt signaling in ThrbPV/PV mice was confirmed by in situ hybridization analysis of Wnt target gene expression in bone during postnatal growth. By contrast, T₃ treatment inhibited Wnt signaling in osteoblastic cells, suggesting that T₃ inhibits the Wnt pathway by facilitating proteasomal degradation of β-catenin and preventing its accumulation in the nucleus. Activation of the Wnt pathway in ThrbPV/PV mice, however, results from a gain of function for TRβPV that stabilizes β-catenin despite the presence of increased thyroid hormone levels. These studies demonstrate novel interactions between T₃ and Wnt signaling pathways in the regulation of skeletal development and bone formation.

Thyroid hormones regulate skeletal development and adult bone maintenance acting directly in chondrocytes and bone-forming osteoblasts and via less well defined indirect pathways (1, 2). Thyroid hormone action is mediated by the nuclear thyroid hormone receptors, TRα and TRβ, which function as 3,5,3′-l-triiodothyronine (T₃)-inducible transcription factors that regulate expression of T₃-responsive target genes (3).

In childhood, thyroid hormone deficiency causes growth retardation and delayed bone age with epiphyseal dysgenesis due to abnormal development of the growth plates resulting in short stature (2). Treatment of hypothyroid children with thyroxine induces a period of rapid catch-up growth and bone maturation (4). By contrast, thyrotoxicosis in children accelerates growth and skeletal maturation but also causes short stature due to early fusion of the growth plates. Premature fusion of the sutures of the skull is also seen in severely affected young children and may lead to craniosynostosis (5). These observations demonstrate sensitivity of the skeleton to disturbances of thyroid status during postnatal growth and indicate the importance of thyroid hormones during bone development.

In adults, thyroid hormone excess causes high bone turnover with uncoupling of the activities of bone-forming osteoblasts and bone-resorbing osteoclasts (6). This results in a net loss of bone in hyperthyroidism leading to osteoporosis and an increased susceptibility to fragility fractures (7–10). In hypothyroidism, there is reduced bone turnover with increased bone mineralization. Population studies indicate that hypothyroid patients also have an increased risk of fracture (7, 8, 10), although the underlying reasons for this have not been established. We recently showed that variation in normal thyroid status in healthy euthyroid postmenopausal women is associated with changes in bone mineral density and fracture risk (11), further demonstrating the sensitivity of the adult skeleton to thyroid status.

The mechanisms of T₃ action in the skeleton have been investigated in Thra and Thrb knock-out mice lacking TRα and TRβ.
TRβ (12–14) and in mice with targeted mutations affecting Thra1 and Thrb (15–17). These studies demonstrated that T₃ action in bone is mediated primarily by TRα and that thyroid hormones stimulate bone formation and mineralization during skeletal growth but increase bone turnover and bone loss in adulthood (2, 13). Analyses of TR mutant mice harboring a PV mutation targeted to either Thra or Thrb have been particularly instructive because of their clear skeletal phenotypes (16–18).

The mutation was originally described in a patient affected with severe resistance to thyroid hormone (RTH) (19). The PV mutation is a C-insertion at codon 448 in the carboxyl-terminal 14 amino acids and generating a mutant TRβ protein that cannot bind T₃ or activate target gene transcription and that acts as a dominant-negative antagonist of wild-type TRs (19, 20). Homozygous ThrbPV/PV mutant mice have a severely impaired pituitary-thyroid axis with markedly elevated circulating thyroid hormone levels (20), whereas Thra1PV/+ heterozygous mice have mild thyroid failure but are systemically euthyroid (21).

ThrbPV/PV mice have advanced endochondral and intramembranous ossification with increased bone mineral deposition during growth, whereas Thra1PV/+ mice have grossly delayed endochondral and intramembranous ossification, reduced bone mineral deposition, and severe growth retardation (16–18). In situ hybridization analysis of skeletal T₃ target gene expression indicated evidence of increased T₃ signaling in ThrbPV/PV bone but impaired T₃ action in the Thra1PV/+ skeleton. Thus, we hypothesized that elevated thyroid hormones in ThrbPV/PV mice drive a phenotype of skeletal thyroid hormone excess via increased stimulation of TRα1 in bone, whereas the phenotype of skeletal thyroid hormone deficiency in Thra1PV/+ mice results from impaired TRα1 activity despite normal circulating thyroid status (18).

Although these studies have resulted in significant advances in our understanding of the molecular mechanisms of T₃ action in bone, the downstream signaling pathways that mediate T₃ responses in the skeleton are poorly understood. To investigate further, we performed microarray studies comparing gene expression in primary osteoblasts from wild-type mice and ThrbPV/PV mice with evidence of increased T₃ signaling in bone. In ThrbPV/PV osteoblasts and in ThrbPV/PV mice in vivo, there was increased activation of canonical Wnt signaling, a pathway that is essential for both skeletal development and the maintenance of adult bone mass (22).

**EXPERIMENTAL PROCEDURES**

**ThrbPV Mice**—Animal studies were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the NCI, National Institutes of Health, Animal Care and Use Committee. Wild-type and homozygous ThrbPV/PV mutant mice were bred and genotyped as described (20).

**Primary Osteoblast Culture**—Primary osteoblasts were prepared from calvaria of 3–5-day-old mice. Calvaria were dissected free of soft tissue and washed in PBS followed by Hanks’ balanced salt solution containing penicillin/streptomycin/neomycin (PSN) (50 µg/ml, 50 µg/ml, and 100 µg/ml, respectively) and amphotericin B (1.5 µg/ml). Washed calvaria were cut into 2–3-mm strips and digested with 0.5% trypsin in PBS containing 4 mM EDTA, PSN, and fungizone. Osteoblasts were released by sequential 30-min digestions with type II collagenase (Worthington Biochemical Corp.). Fibroblasts released from the first two digestions were discarded, and osteoblasts from digestions 3–5 were pooled, centrifuged, and resuspended in α-minimum essential medium supplemented with 10% heat-inactivated FBS, PSN, and amphotericin B. After 4–5 h, the medium was replaced, and adherent cells were cultured for 7 days in differentiation-permissive α-minimum essential medium containing 10% heat-inactivated FBS or 10% thyroid hormone-deprived heat-inactivated FBS supplemented with PSN, amphotericin B, ascorbic acid (50 µg/ml), and α-glycerophosphate (5 mM) in the absence or presence of T₃ (100 nM).

**Microarray**—The beaded mouse arrays contained 30,336 cDNAs. Hybridization, scanning, and image analysis were performed as described (www.nhgri.nih.gov/DIR/microarray) (23–26). Fluorescence-labeled cDNA was synthesized from 20 µg of pooled calvarial osteoblast total RNA obtained from six wild-type and seven ThrbPV/PV mice or from individual mice. Labeled cDNA was synthesized by oligo(dT)-primed polymerization in the presence of aminooxy-1,4-DUTP (Amersham Biosciences) and coupled to either Cy-3 or Cy-5. Image analysis was performed using the DeArray software (Signal Analytics, Vienna, VA) (23, 26). The red and green channel fluorescent images from one array constituted the raw data from which differential gene expression ratios were calculated. Raw data values were thresholded to have values equal to the largest non-negative value and log-transformed. Red-to-green intensity ratios for all genes were determined, and values within each array were normalized to a median value of 1. Ratios associated with quality factors between 0.5 and 1.0 were included for further analysis, and only genes with ratios of ≥2 or <0.5 in osteoblasts from ThrbPV/PV mice relative to wild type were considered to be significantly up- or down-regulated.

**qRT-PCR**—Total RNA was extracted from primary osteoblasts using TRIzol (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed using a SYBR Green quantitative RT-PCR kit (Sigma-Aldrich) or QuantiTect quantitative RT-PCR kit (Qiagen) according to the manufacturers’ instructions and a LightCycler thermal cycler (Roche Applied Science, Mannheim, Germany). Briefly, 2.5 µl of forward primer (2 µM) and 2 µl of reverse primer (2 µM) were added to 15 µl of SYBR Green enzyme reaction mix or QuantiTect RT reaction mix. The cycles were: 55 °C for 30 min; 95 °C for 30 s; 95 °C for 15 s; 58 °C for 30 s; and 72 °C for 30 s; 65–95 °C with a heating rate of 0.1 °C/s; and a cooling step to 40 °C. Primer sequences used were: procollagen type I (antisense); mouse-related RAS viral oncogene homolog 2 (antisense) and 5′-GCCATCTCGTCTGTTCACTCTGA-3′ (sense) and 5′-TGCCCGTCTGCTGTTCACTCTGA-3′ (antisense); integrin, α11 (ltga11) 5′-AGCTTCTACCTGTTGGGAA-AC-3′ (sense) and 5′-GAGATCTCAAATGTGACCTGGC-3′ (antisense); mouse-related RS viral oncogene homolog 2 (Rras2) 5′-GCCCCGGCTGGACATTGGATA-3′ (sense) and 5′-CCGTTCTTTACTCTGAGAATC-3′ (antisense); cyclin D1 (Cnd1) 5′-CTGCAATATGGAATCTGGTTG-3′ (sense) and 5′-TTCATCTTGTGGACAGG-3′ (antisense); β-catenin (Ctnnb1) 5′-AGCCCGAGATGGCCACAGA-3′ (sense) and 5′-
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AAGGGCAAGGTTCCGAATCCAA-3’ (antisense); Lrp5 5’-AAGGTTCTGGAACCACTCAGT-3’ (sense) and 5’-TGATCGTCTTGAGGCTGACATCAGT-3’ (antisense); Lrp6 5’-TGATCGTCTTGAGGCTGACATCAGT-3’ (antisense); and 5’-GCAC-AAGGGTTGCTGTCTGACT-3’ (antisense); and 5’-GTCCTCAGTGTAGCCCAAG-3’ (antisense).

Wnt Signaling Pathway PCR Array—A Wnt signaling pathway RT2 Profiler PCR array was used according to the manufacturer’s instructions (SABiosciences, Frederick, MD). Briefly, primary osteoblast total RNA obtained from two wild-type and two homozygous TRβPV/PV mutant littermate mice was extracted using TRIzol, and 1 μg of RNA was used to synthesize wild-type and TRβPV/PV cDNA using SuperScript II reverse transcriptase. cDNA was used as a template for Wnt signaling pathway PCR arrays and experiments were performed in duplicate. Arrays contained five reference housekeeping genes for normalization of gene expression. qRT-PCR was performed using a Bio-Rad IQ5 thermal cycler. The cycles were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 35 s, and 72 °C for 30 s.

In Situ Hybridization—Expression of Rankl, Runx2, and Wnt4 mRNAs in growth plate chondrocytes and osteoblasts was determined by in situ hybridization of tissue sections obtained from postnatal days 0 and 14 mice. A bacterial neo-mycin resistance gene cRNA probe (Roche Applied Science, Lewes, Sussex, UK) was used as a negative control for all hybridizations. Mouse Rankl (nucleotides 695–1110; GenBank accession number NM_011613.3), Runx2 (nucleotides 1350–1781; GenBank accession number NM_009820.2), and Wnt4 (nucleotides 161–562; GenBank accession number NM_009523.2) partial cDNAs were isolated by RT-PCR from chondrogenic ATDC5 cells (27) using the following primers: Rankl, forward, 5’-GTGATCTCTCAGGTGCCAACA-3’, reverse, 5’-CCGCTCCAGTCTCTG-3’; Runx2, forward, 5’-TTGCTCCAAAGCTTACCAA-3’, reverse, 5’-GCCCAAACAGACTCATCCAT-3’; Wnt4, forward, 5’-CGAGAGAGACGTGCGAGAAA-3’, reverse, 5’-CCAATCAATAACGTT-3’. PCR products were subcloned into pGEM-T Easy vector (Promega, Southampton, Hampshire, UK) and sequenced. Rankl and Wnt4 constructs were linearized with SpeI, and the Runx2 construct was linearized with NcoI before digoxygenin-labeled cRNA probes were synthesized using T7 and SP6 RNA polymerases, respectively (Roche Applied Science). In situ hybridizations using alkaline phosphatase-labeled probes were performed on 3-μm deparaffinized sections as described in detail (17, 28, 29).

Transient Transfection and Adenovirus Infection—MC3T3 and UMR106 cells (1.5 × 10^5 cells/well of a 6-well plate) were plated 18–24 h before transfection in either α-minimum essential medium (MC3T3) or DMEM (UMR106) supplemented with 10% thyroid hormone-deprived FBS and PSN. Cells were transfected with a β-catenin-TCF4-responsive TOP-Flash reporter plasmid (TCF4; 1 μg) or thyroid hormone response element reporter plasmid (PAL-Luc; 1 μg) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. At the same time, adenovirus encoding TRα1 or TRβ1 was infected into cells at a multiplicity of infection of 10. After a 3-h incubation, medium was replaced by fresh 10% thyroid hormone-deprived medium with or without T3 (100 nM). Cells were lysed after 24 h with 3 × cell lysis buffer (Pharmingen), and luciferase activity was determined according to the manufacturer’s protocol using a Victor3 multilabel counter with dual-injection capability (PerkinElmer Life Sciences). Luciferase values were standardized to protein concentration.

Western Blotting—Western blot analysis of β-catenin and phospho-β-catenin was performed as described (30, 31). UMR106 cells were seeded in 6-mm wells (5 × 10^5 cells/well) in DMEM supplemented with 10% thyroid hormone-deprived FBS. After 24 h, the medium was changed to Opti-MEM (Invitrogen) prior to adenovirus infection. Cells were infected at a multiplicity of infection of 10 with adenovirus encoding FLAG-tagged TRβ1 or TRβ1PV. After 3 h, T3 (100 nM) was added, and cells were lysed 6 or 24 h later in 1 × lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) containing proteinase inhibitor (Complete Mini EDTA-free; Roche Applied Science) and protein phosphatase inhibitor cocktails (Thermo Scientific). Protein concentrations were determined by Bradford assay (Pierce), and 20 μg of total protein was used for Western blotting with the following antibodies: anti-β-catenin (1:1000 dilution, antibody 9562, Cell Signaling), anti-phospho-β-catenin (Ser552) (1:1000 dilution, antibody 9566, Cell Signaling), anti-GAPDH (1:1000 dilution, antibody 2118, Cell Signaling), and anti-TRβ (J53) (32). Protein expression was detected by enhanced chemiluminescence and quantified using ImageJ (rsb.info.nih.gov/ij/index.html) (30).

Statistics—Data were expressed as mean ± S.E. The differences between groups were examined for statistical significance using one-way analysis of variance followed by Tukey’s multiple comparison post hoc test or by two-tailed Student’s t test as appropriate. p values <0.05 were considered significant.

RESULTS

Microarray Analysis—Microarrays comprising 30,336 cDNAs were used to compare gene expression profiles between wild-type and TRβPV/PV mice. Only genes up-regulated at least 2-fold or down-regulated at least 50% in mutants as compared with wild-type mice were considered to be of potential biological significance. Of 192 differentially expressed genes, 85 (44%) were up-regulated and 107 (56%) were down-regulated in TRβPV/PV mice (supplemental Fig. 1). Empirical Bayes analysis indicated >97% likelihood that differences in gene expression resulted from true biological variation rather than by chance. To obtain deeper insight into the signaling pathways in osteoblasts associated with advanced skeletal development in TRβPV/PV mice (16, 17), we classified differentially expressed genes according to function using controlled vocabulary gene ontology terms with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics database (33) and Expression Analysis Systemic Explorer (EASE) software (david.niaid.nih.gov/david/ease.htm) (34). Using this method, seven functional categories were identified in which to classify the 192 differentially expressed genes: focal adhesion pathway; signal transduction; enzyme regulation; development; transcriptional regulation; regulation of biological processes; and structural molecules (supplemental Tables 1–7).
Functional Pathway Analysis and Validation of Microarray Data—Investigation of classification categories using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Resource revealed that nine differentially expressed genes act within the focal adhesion pathway (supplemental Table 1). This network converges at contact points between the cell membrane and extracellular matrix where actin filaments anchor to integrin receptors via junctional protein complexes. These focal adhesion contact points contain both structural and signaling proteins that mediate intracellular responses to extracellular signals and regulate processes such as cell shape and motility, cell proliferation, differentiation and survival, and gene expression. Various extracellular signals that include mechanical stress, kinases and phosphatases, matrix ligands, hormones, and growth factors all impact on focal adhesion networks and demonstrate the fundamental significance of the pathway (35, 36).

To substantiate results from microarray experiments, the expression of four focal adhesion pathway genes, Col1a1, Itga11, Rras2, and Ccnd1, was investigated by qRT-PCR in independent cultures of wild-type and ThrβPV/PV primary osteoblasts. In these studies, Col1a1 mRNA expression was reduced by 50% in ThrβPV/PV osteoblasts as compared with wild type, and Itga11 expression was decreased by 75%, whereas Rras2 expression increased 1.4-fold and Ccnd1 mRNA increased 4.7-fold (Fig. 1). These changes were consistent with the 80 and 90% reductions in Col1a1 and Itga11 expression and 2.8- and 2.5-fold increases in Rras2 and Ccnd1 expression determined in microarray studies. Furthermore, overexpression of Ccnd1 has been reported in human thyroid cancer (37–39) and in thyroid and pituitary glands of ThrβPV/PV mice, which are susceptible to both thyroid cancer and pituitary thyrotroph tumors (24, 40). Taken together, these studies validate the microarray studies and are consistent with previous studies in different tissues from ThrβPV/PV mice.

Expression of Wnt/β-Catenin Pathway Genes in TRβPV/PV Osteoblasts—Increased expression of Ccnd1 (2.5-fold) and the Drosophila homolog Frizzled 7 (Fzd7) (2.35-fold) in ThrβPV/PV osteoblasts (supplemental Table 4) implicates
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Wnt/β-catenin signaling, a key pathway that regulates bone mass (41), as a target for thyroid hormones in osteoblasts. Ccnd1 encodes the cell cycle regulator cyclin D1 and is a downstream target gene of Wnt/β-catenin signaling (42), and Fzd7 encodes a seven-transmembrane G-protein-coupled receptor that binds Wnt and mediates activation of the Wnt/β-catenin signaling cascade (43). Thus, we used a mouse Wnt signaling pathway PCR array to investigate expression of 84 genes related to Wnt-mediated signal transduction in ThrbPV/PV primary osteoblasts. 34 Wnt pathway genes were differentially expressed in ThrbPV/PV primary osteoblasts as compared with wild type (Table 1). Ccnd1 expression was increased 3.66-fold and Fzd7 expression was increased 1.83-fold in ThrbPV/PV osteoblasts, demonstrating consistency with microarray analyses. Overall, expression of 12 Wnt pathway genes was increased in ThrbPV/PV osteoblasts, whereas 22 genes were down-regulated.

The canonical Wnt/β-catenin pathway is complex. Its activity is regulated by an interplay involving at least 19 Wnt ligands, 10 Frizzled receptors, lipoprotein-related receptors 5 and 6 (LRP5, LRP6), and various soluble inhibitors that act at different sites in the pathway to regulate its activity (22, 44, 45). Transduction of the Wnt signal following activation of the LRP/Frizzled co-receptor complex involves several intracellular proteins and enzymes that ultimately lead to dephosphorylation of the transcription factor β-catenin. This prevents sequestration and degradation of β-catenin in the cytoplasm, resulting in its translocation to the nucleus, where it acts as a specific co-activator of Wnt-dependent transcription factors (Tcf/Lef) family (22, 44, 45). In this context, therefore, it is not possible to predict the overall functional response of the Wnt pathway to external regulating factors such as thyroid hormones simply by analyzing changes in the expression patterns of mRNAs encoding the various Wnt pathway components.

**Table 1**

Analysis of RT² Profiler™ PCR array mouse Wnt signaling pathway

| GenBank accession number | Symbol | Gene description | Fold change |
|--------------------------|--------|------------------|-------------|
| NM_019715                | Tle2   | Transducin-like enhancer of split 2, homolog of Drosophila E(spl) | −1.88       |
| NM_023653                | Wnt2   | Wingless-related MMTV integration site 2 | 2.22        |
| NM_009520                | Wnt2b  | Wingless-related MMTV integration site 2b | −2.30       |
| NM_009525                | Wnt5b  | Wingless-related MMTV integration site 5b | −2.22       |
| NM_009527                | Wnt7a  | Wingless-related MMTV integration site 7a | 8.57        |
| NM_011720                | Wnt8b  | Wingless-related MMTV integration site 8b | 2.97        |
| NM_009519                | Wnt11  | Wingless-related MMTV integration site 11 | 2.81        |
| NM_053116                | Wnt16  | Wingless-related MMTV integration site 16 | −6.54       |

Negative regulation of the Wnt receptor

| GenBank accession number | Symbol | Gene description | Fold change |
|--------------------------|--------|------------------|-------------|
| NM_010051                | Dkk1   | Dickkopf homolog 1 (Xenopus laevis) | 7.11        |
| NM_013834                | sFRP1  | Secreted frizzled-related sequence protein 1 | −39.95      |
| NM_009164                | sFRP2  | Secreted frizzled-related sequence protein 2 | −390.72     |
| NM_016687                | sFRP4  | Secreted frizzled-related sequence protein 4 | −5.43       |

Other genes related to growth and proliferation

| GenBank accession number | Symbol | Gene description | Fold change |
|--------------------------|--------|------------------|-------------|
| NM_080043                | Frat1  | Frequently rearranged T-cell lymphomas | −3.68       |
| NM_007631                | Ccnd1  | Cyclin D1 | 3.66        |
| NM_009829                | Ccnd2  | Cyclin D2 | 2.64        |

Transcription factors

| GenBank accession number | Symbol | Gene description | Fold change |
|--------------------------|--------|------------------|-------------|
| NM_008238                | Foxn1  | Forkhead box N1 | 4.14        |
| NM_007614                | Ctnnb1 | Catenin (cadherin-associated protein), β 1 | −1.84       |
| NM_011098                | Ptx2   | Paired-like homeodomain transcription factor 2 | −2.38       |
| NM_009331                | Tcf7   | Transcription factor 7, T-cell specific | −2.07       |

Protein kinase activity

| GenBank accession number | Symbol | Gene description | Fold change |
|--------------------------|--------|------------------|-------------|
| NM_007631                | Ccnd1  | Cyclin D1 | 3.66        |

Other Wnt signaling pathway-related genes

| GenBank accession number | Symbol | Gene description | Fold change |
|--------------------------|--------|------------------|-------------|
| NM_008045                | Fshb   | Follicle-stimulating hormone beta | 2.69        |
| NM_021457                | Fzd1   | Frizzled homolog 1 (Drosophila) | −3.41       |
| NM_008055                | Fzd4   | Frizzled homolog 4 (Drosophila) | −2.58       |
| NM_008056                | Fzd6   | Frizzled homolog 6 (Drosophila) | −2.11       |
| NM_008057                | Fzd7   | Frizzled homolog 7 (Drosophila) | 1.83        |
| NM_027280                | Nkd1   | Naked cuticle homolog (Drosophila) | −6.68       |
| NM_023638                | Porcn  | Porcupine homolog (Drosophila) | −5.86       |
| NM_011915                | Wif1   | Wnt inhibitory factor 1 | −6.15       |
microarray, RT-PCR, and Wnt PCR array studies suggests the Wnt/β-catenin pathway may be activated in bone in ThrbPV/PV mice. To investigate this possibility, we determined expression of the Rankl (receptor activator of NFκB ligand) and Runx2 (Runt-related transcription factor-2) Wnt target genes in bones obtained from neonatal and 2-week-old ThrbPV/PV mice by in situ hybridization. Expression of Rankl is reduced following activation of Wnt signaling (46), whereas expression of Runx2 is increased (47).

In 2-week-old ThrbPV/PV mice, Rankl expression was markedly reduced or absent in osteoblasts lining trabecular bone surfaces as compared with expression that was readily detectable in wild-type littermates (Fig. 2A). In neonatal mice, Runx2 expression was increased markedly in perichondrial cells surrounding the developing growth plate in ThrbPV/PV mice as compared with wild-type littermates (Fig. 2B). These findings are consistent with increased activation of the Wnt/β-catenin pathway in bone in ThrbPV/PV mice during endochondral ossification and postnatal growth.

In previous studies, Wang et al. (48, 49) found that T3 activates Wnt signaling in growth plate chondrocytes. They demonstrated increased expression of β-catenin (Ctnnb1) and its target gene Runx2, as well as increased expression of the activating Wnt ligand Wnt4 following T3 treatment.

To investigate further, we determined expression of Wnt4 in bones obtained from 2-week-old ThrbPV/PV mice. In ThrbPV/PV mice, Wnt4 expression was markedly reduced or absent in both growth plate chondrocytes and osteoblasts lining trabecular bone surfaces as compared with expression that was readily detectable in wild-type littermates (Fig. 2C). These data are consistent with the dominant-negative activity of mutant TRβPV protein (19, 20) and with previous studies indicating that Wnt4 expression is increased in chondrocytes in response to T3 (48, 49). Nevertheless, the findings reveal an overall discrepancy in ThrbPV/PV mice, in which increased Wnt/β-catenin activity in bone and cartilage is accompanied by reduced expression of the activating ligand Wnt4.

**Regulation of β-Catenin Signaling in Osteoblasts by T3**—To investigate whether T3 influences activity of the Wnt/β-catenin pathway, we determined whether T3 regulated expression of a β-catenin/Tcf/Lef-responsive reporter construct (50, 51) in transfected osteoblasts infected with adenovirus expressing TRα1 or TRβ1 (Figs. 3 and 4). Transfection of rat UMR106 osteosarcoma cells with a well characterized thyroid hormone response element reporter (PAL-Luc) (52, 53) in the presence of TRα1, but not TRβ1, resulted in unliganded apoTRα-mediated repression of reporter gene activity in the absence of T3, as reported previously (53). In the presence of T3, TRα1 and TRβ1 mediated 8.2- and 12.5-fold increases in reporter gene activity, respectively (Fig. 3), thus demonstrating functional integrity of TR signaling. By contrast, T3 treatment repressed activity of the β-catenin/Tcf/Lef reporter in UMR106 cells co-transfected with TRα1 (expression reduced 2.4-fold) or TRβ1 (expression reduced 5.2-fold). Treatment with T3 in the absence of co-transfected TR did not affect β-catenin/Tcf/Lef reporter activity, and apoTR in the absence of T3 also did not affect reporter gene expression. Thus, β-catenin signaling in UMR106 cells was inhibited by T3 by a TR-dependent mechanism (Fig. 3). Experiments in mouse osteoblastic MC3T3 cells similarly resulted in repression of β-catenin/Tcf/Lef reporter gene activity by T3 by a TR-dependent mechanism, with equivalent results also obtained using adenovirus expressing either TRα1 or TRβ1 (Fig. 4). Thus, T3 inhibits activity of the Wnt/β-catenin signaling pathway in osteoblasts.

**TRβPV Regulates β-Catenin stability in Osteoblasts**—In microarray studies, β-catenin mRNA expression did not differ
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between Thrb\textsuperscript{PV/PV} and wild-type osteoblasts (data not shown), whereas in Wnt PCR array experiments, expression of β-catenin was reduced in Thrb\textsuperscript{PV/PV} osteoblasts by 2.38-fold (60%) (Table 1). However, in independent RT-PCR analyses, the level of β-catenin mRNA did not differ in Thrb\textsuperscript{PV/PV} osteoblasts, suggesting that changes in β-catenin mRNA expression do not account for increased activation of the canonical Wnt pathway in Thrb\textsuperscript{PV/PV} osteoblasts (Fig. 5A). Similarly, expression of Lrp5 and Lrp6 mRNAs did not differ in Thrb\textsuperscript{PV/PV} osteoblasts as compared with wild-type in RT-PCR studies, suggesting that altered expression of these Wnt co-receptors also does not account for increased Wnt signaling in Thrb\textsuperscript{PV/PV} osteoblasts (supplemental Fig. 2).

To investigate in further detail, we determined the effect of T\textsubscript{3} on endogenous β-catenin and TRβ protein in UMR106 osteoblastic cells in the absence and presence of wild-type receptor or mutant TRβ\textsuperscript{PV} (Fig. 5B). Wild-type TRβ and
β-catenin protein concentrations were decreased following T3 treatment for 6 and 24 h, consistent with the previously reported T3-induced proteasome-mediated degradation of TRα in other cell types and the physical interaction between unliganded TRα and β-catenin that is dissociated by T3 (54). By contrast, TRβPV levels were not affected by T3 as the mutant protein cannot bind ligand. Accordingly, there was no effect of T3 treatment on β-catenin levels in cells lacking wild-type or mutant TR. Furthermore, β-catenin was stabilized in TRβPV-expressing UMR106 cells (Fig. 5B). Taken together, these data demonstrate a gain of function for TRβPV in osteoblasts that stabilizes β-catenin in the presence of T3.

DISCUSSION

These studies demonstrate increased activity of the canonical Wnt/β-catenin pathway in the skeleton of ThrbPV/PV mice in vivo and in ThrbPV/PV osteoblasts in vitro. By contrast, T3 treatment of osteoblastic cells results in inhibition of Wnt/β-catenin signaling.

Activation of Wnt signaling leads to a postnatal increase in bone mass that is mediated by osteoblasts, whereas deletion of the Wnt co-receptor Lrp5 results in reduced bone mass (55, 56). Similarly, deletion of sclerostin, a secreted Wnt inhibitor in bone, results in a phenotype of high bone mass (57). Thus, activation of the Wnt pathway stimulates osteoblastic bone formation during growth and accrual of bone mass in adults, whereas loss-of-function mutations in the Wnt pathway lead to low bone mass due to defective osteoblast proliferation and maturation (22, 58). The finding of increased activation of the Wnt/β-catenin pathway in the skeleton in ThrbPV/PV mice in vivo and in ThrbPV/PV osteoblasts is consistent with the established role of the Wnt pathway in bone (59) and the phenotype of advanced ossification in ThrbPV/PV mice (17).

In the context of our previous hypothesis that advanced ossification in ThrbPV/PV mice results from increased activation of the predominantly expressed wild-type TRα in skeletal cells (18), the finding of increased Wnt signaling activity in ThrbPV/PV bone suggested that T3 would stimulate Wnt/β-catenin signaling in osteoblasts. However, in two different osteoblastic cell lines in the presence of either TRα or TRβ, T3 inhibited activity of a β-catenin/Tcf/Lef reporter construct in a TR-dependent manner.

In previous studies, we showed that β-catenin activity was also increased in thyroid tumors that develop in ThrbPV/PV mice with increasing age (40). In studies to investigate the molecular basis for increased β-catenin activity in ThrbPV/PV thyroid tissue, we demonstrated that unliganded wild-type TRβ and TRβPV proteins physically interact with β-catenin, leading to its stabilization and an increase in Wnt signaling (54). The association between wild-type TRβ and β-catenin was disrupted by T3, leading to targeted degradation of...
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\(\beta\)-catenin by the proteasome. T\(_3\) and TR\(\beta\)-dependent degradation of \(\beta\)-catenin resulted in repression of Wnt signaling by thyroid hormone. By contrast, the interaction between mutant TR\(\beta\)PV and \(\beta\)-catenin was not disrupted by T\(_3\), and thyroid hormone treatment had no effect on the increased activation of Wnt signaling in TR\(\beta\)PV-expressing cells (54). These data are consistent with the current findings of increased Wnt/\(\beta\)-catenin signaling in bone in Thrb\(PV/PV\) mice in vivo and the stabilization of \(\beta\)-catenin protein in osteoblasts expressing TR\(\beta\)PV. They are also consistent with the inhibition of Wnt signaling observed in osteoblasts treated with T\(_3\) and the degradation of \(\beta\)-catenin in osteoblasts expressing wild-type TR\(\beta\).

Nevertheless, our previous studies in mutant mice indicate that T\(_3\) action in bone is mediated primarily by TR\(\alpha\), although both TR\(\alpha\) and TR\(\beta\) are expressed in skeletal cells (1, 2, 13). The finding that Wnt/\(\beta\)-catenin signaling was inhibited equally by T\(_3\) in the presence of either TR\(\alpha\) or TR\(\beta\) (Figs. 3 and 4) suggests that, like TR\(\beta\) (54), TR\(\alpha\) is also able to associate with \(\beta\)-catenin. By analogy, it could be expected that Wnt/\(\beta\)-catenin signaling activity would be increased in the skeleton of Thra1\(PV/PV\) mice. Although this has not been investigated, the finding of an opposite phenotype of delayed ossification, reduced bone mineral deposition, and growth retardation in Thra1\(PV/PV\) mice as compared with Thrb\(PV/PV\) mice (16, 17) is not consistent with such a hypothesis. One possibility to account for the difference in skeletal phenotypes between Thra1\(PV/PV\) and Thrb\(PV/PV\) mice is that TR\(\alpha\)PV differs from TR\(\beta\)PV and might not be able to interact with and stabilize \(\beta\)-catenin and thus be unable to increase activity of the Wnt pathway. Alternatively, complete blockade of thyroid hormone signaling in Thra1\(PV/PV\) bone that results in severe developmental delay in the skeleton is very likely to override any effect of an increase in Wnt signaling activity that might result from stabilization of \(\beta\)-catenin by interaction with mutant TR\(\alpha\)PV protein. Studies of the Wnt/\(\beta\)-catenin pathway in Thra1\(PV/PV\) mice and osteoblasts will be required to determine which of these possibilities is correct.

Taken together, these considerations suggest that the skeletal phenotype of delayed ossification in Thra1\(PV/PV\) mice results solely from deficient thyroid hormone action in bone, whereas the current studies demonstrate that the advanced ossification in Thrb\(PV/PV\) mice results from activation of the Wnt signaling pathway in bone as well as from increased T\(_3\) action. Although we show that T\(_3\) normally inhibits activity of the Wnt pathway in osteoblasts, this paradoxical finding results from a gain of function of the mutant TR\(\beta\)PV protein, which is able to associate with and stabilize \(\beta\)-catenin in the presence of T\(_3\) (54). In addition to this gain-of-function activity, the reduced levels of Wnt\(\alpha\) expression in Thrb\(PV/PV\) chondrocytes and osteoblasts in vivo suggest that the mutant TR\(\beta\)PV protein also exhibits dominant-negative activity in the skeleton to inhibit previously described stimulatory effects of T\(_3\) on Wnt\(\alpha\) expression (48, 49).

Overall, the skeletal consequences of the TR\(\beta\) PV mutation are a complex balance resulting from a combination of (i) increased T\(_3\) action mediated by the actions of elevated thyroid hormones on TR\(\alpha\) in bone, (ii) a gain of function of the mutant TR\(\beta\)PV protein to stabilize \(\beta\)-catenin and activate Wnt signal-

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