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Genetic Evidence that Thyroid Hormone is Indispensable for Prepubertal IGF-I Expression and Bone Acquisition in Mice

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SUMMARY

Understanding how bone growth is regulated by hormonal and mechanical factors during early growth periods is important for optimizing the attainment of peak bone mass to prevent or postpone the occurrence of fragility fractures later in life. Using genetic mouse models that are deficient in thyroid hormone (TH) (*Tshr*^{-/-} and *Duox2*^{-/-}), growth hormone (GH) (*Ghrhr*^{lit/lit}) or both (*Tshr*^{-/-}; *Ghrhr*^{lit/lit}), we demonstrate that there is an important period prior to puberty when the effects of GH are surprisingly small and TH plays a critical role in the regulation of skeletal growth. Daily administration of T3/T4 during days 5 to 14, the time when serum levels of T3 increase rapidly in mice, rescued the skeletal deficit in TH-deficient mice but not in mice lacking both TH and GH. However, treatment of double-mutant mice with both GH and T3/T4 rescued the bone density deficit. Increased body fat in the TH-deficient as well as TH/GH double mutant mice was rescued by T3/T4 treatment during days 5–14. *In vitro* studies in osteoblasts revealed that T3 in the presence of TH receptor (TR) α bound to a TH response element in intron 1 of the IGF-I gene to stimulate transcription. *In vivo* studies using TR α and TR β knockout mice revealed evidence for differential regulation of IGF-I expression by the two receptors. Furthermore, blockade of IGF-I action partially inhibited the biological effects of TH, thus suggesting that both IGF-I-dependent and independent mechanisms contribute to TH effects on prepubertal bone acquisition.

Keywords

Growth Hormone; Osteoblasts; Thyroid hormone receptor alpha; Thyroid hormone receptor beta; Dual oxidase-2, Hypothyroidism; Acid labile subunit; IGF binding protein

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INTRODUCTION

Childhood and adolescence is a particularly important time to maximize bone acquisition since the skeleton undergoes rapid changes owing to the processes of growth, modeling and remodeling (1,2). Peak bone mass is attained in young adulthood, remains relatively constant in early adulthood, and then gradually declines in aging men and women (3). The achievement of an optimal bone mass is recognized as a primary health goal during early growth periods since it is widely understood that the risk of fragility fractures in old age has its origin during growth (4). For example, it has been reported that 60% of the risk of osteoporosis can be explained by the amount of bone mineral acquired by early adulthood (5). Therefore, information learned from studies on the mechanisms regulating bone acquisition during postnatal growth periods is of considerable importance in developing strategies to postpone or reduce the risk of osteoporotic fractures. With regard to the potential signaling molecules that contribute to skeletal growth, the findings from a variety of conditional knockout (KO) models and clinical studies of patients with mutations in genes that regulate insulin-like growth factor (IGF)-I action have illustrated a critical role for IGF-I in bone acquisition during postnatal growth (2,5–9). In terms of the mechanisms regulating IGF-I action, it is known since the discovery of IGF-I as a sulfation factor and somatomedin, that a much of IGF-I action is regulated by growth hormone (GH)-dependent mechanisms (10,11). This conclusion is based on findings that disruption of production and/or actions of GH results in impaired skeletal growth and markedly reduced serum IGF-I levels as revealed in both animal and human clinical studies (7,12–14).

Twin and family studies have demonstrated a strong genetic component in the determination of peak bone mass (15,16). Consistent with the human data, animal studies have revealed there is considerable variation in peak bone mass between inbred strains of both mice and rats, and that these differences are largely attributable to small differences in their genetic background (15–17). Studies have shown that bone mineral density (BMD) has a relatively high heritability, which ranges from 0.5 to 0.9 in human and mouse (15–17). In order to identify the critical developmental period when the differences in bone mass accrual occur, we have compared the relative gain in various parameters at several time points from day 7 to day 56 in C57BL/6J and C3H/HeJ inbred strains of mice, and have demonstrated a 70% difference in femoral BMD (18). These studies revealed that days 7 to 14 of the prepubertal period and days 23 to 31 of the pubertal period represent critical time points for skeletal growth as well as increases in serum IGF-I levels in mice (18). In subsequent studies using mice with inactivation of the IGF-I gene, GH deficiency and deletion of the GH receptor, it was determined that there is an important critical period during pre-pubertal growth when the effects of GH are small and IGF-I remains an important regulator (7). Accordingly, poor growth responses to GH treatment during the prepubertal growth period are frequently seen in clinical studies using recombinant human GH therapy in children with certain growth disorders (19,20). While these and other data have provided irrefutable evidence for the involvement of other factors besides GH in prepubertal bone acquisition, GH-independent mechanisms that regulate IGF-I expression and bone acquisition remain to be elucidated.

In this study, we evaluated the role of thyroid hormone (TH) in regulating prepubertal bone acquisition, since childhood hypothyroidism causes growth failure and since targeted disruption of TH receptor in mice influences skeletal growth (21–24). Furthermore, studies by Bassett et al. (25) have shown using 3,5,3'-L-triiodothyronine (T3) receptor alpha (TR α) knock-in mutant mice that there is a discrete period of time during post-natal growth in which TR α is essential for the actions of TH. In addition to the skeletal phenotype, we also evaluated the consequence of TH deficiency on the fat phenotype because of growing awareness of a bone-fat connection (26) and because TH effects on fat metabolism is poorly understood despite the well known effect of TH on the metabolic rate and heat production

(27). Our data using mutant mouse models with TH and/or GH deficiency provide direct evidence that TH deficiency produces a greater skeletal deficit than GH deficiency during the prepubertal growth period. While T3/T4 treatment rescues the skeletal deficit in TH deficient mice, administration of both GH and T3 are required during the prepubertal growth period to rescue the skeletal deficit in the GH/TH deficient double-mutant mice. By contrast, increased body fat in the TH-deficient as well as TH/GH double mutant mice is rescued by T3/T4 treatment.

MATERIALS AND METHODS

Chemicals, recombinant proteins and biological reagents

T3 and T4 were purchased from Sigma (Saint Louis, MO). Recombinant human insulin-like growth factor binding protein-4 (IGFBP4) was purified from *E. Coli* as previously reported (28). Plasmid pTAL-luc-IGF-I was generated by inserting a DNA fragment from intron 1 of IGF-I gene (2113 to 2152 downstream of the transcription start site) containing a putative TH response element (TRE) in front of the TK minimal promoter of pTAL (Clontech, Mountain View, CA). Small interference RNA (siRNA) specifically against both TR 1 and TR 2, and non-specific control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Adenoviruses expressing TR 1, TR 2 or -Gal were generated as described (29).

Mouse models

Ghrhr^{lit/+} heterozygous mice with a point mutation in GH releasing hormone receptor and *Tshr^{-/+}* heterozygous mice with a point mutation in the coding region of the thyroid stimulating hormone receptor gene were purchased from the Jackson laboratory (Bar Harbor, Maine). Dual oxidase-2 mutant mice (*Duox2^{-/-}*) with hypothyroidism due to a missense mutation in *Duox2* were maintained at Jackson Laboratory as described (30). *Duox2* is an enzyme that is involved in the biosynthesis of THs by generating H₂O₂ required for organification of iodine. Homozygous *Duox2* mutant mice are dwarf, hypothyroid (serum T4 about one-tenth of the controls) and hearing impaired (30). Colonies of TR 0/0 mutant mice harboring a targeted deletion of all transcripts from the TR locus (31) and TR mutant mice harboring targeted deletion of both TR 1 and TR 2 mRNAs (32) were maintained at Imperial College, London, U.K. Mice with a conditional IGF-I KO in cells of osteoblastic lineage were reported previously (6). DNA extracted from tail snips were used for PCR-based genotyping. Newborn C57BL/6J mice with hypothyroidism were generated by treating pregnant mice with 0.05% methimazole in drinking water, as described previously (33). Both male and female mice in approximately equal number were used and the data from both genders were pooled for analyses. All mice were housed at the Jerry L. Pettis Memorial VA Medical Center Veterinary Medical Unit (Loma Linda, CA), Jackson Laboratory (Bar Harbor, ME) or Imperial College (London, U.K.) under standard approved laboratory conditions with controlled illumination (14 hours light, 10 hours dark), temperature (22°C) and unrestricted food and water. All of the procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the Jerry L Pettis Memorial VA Medical Center, Jackson Laboratory or Imperial College.

Evaluation of bone phenotypes

Total bone mineral content (BMC) and BMD were measured by dual-energy X-ray absorptiometry with a PIXImus instrument (LunarCorp., Madison, WI) as described previously (7,12). The volumetric BMD and geometric parameters at the mid-diaphysis of the femur isolated from 3- and 21-day age of mice were determined by Peripheral Quantitative Computed Tomography (pQCT; Norland Stratec XCT 960M, Stratec Medzizintechnik GmbH, Madison, WI) as reported (7,12). Trabecular bone

microarchitecture of the femur isolated from 21 day old mice were assessed by μ -CT (VIVA CT40, Scanco). The femurs were scanned by X-ray (55–70 kVp volts). The voxel size was 10.5 microns. Reconstruction analysis was performed with SCANCO software (SCANCO Medical, Bruttisellen, Switzerland). A fixed section of 1.8 mm starting at 0.36 mm proximal to the growth plate was analyzed for trabecular measurements using SCANCO software.

Serum measurements

IGF-I was measured by RIA after removing IGF binding proteins using an acid gel filtration protocol involving a Bio Spin method (34). T3 levels were measured by a solid phase RIA using a Coat-A-Count Total T3 kit from Diagnostic Products Corporation (Los Angeles, CA).

Cell culture

Primary osteoblasts were isolated from the calvariae of 2 to 4 day old C57BL/6J mice using a modified sequential digestion protocol described previously (35,36). Primary calvarial osteoblasts and MC3T3-E1 cells were plated at $1.5 \times 10^4/\text{cm}^2$ ($1.5 \times 10^5/\text{well}$) in 6-well culture plates in α -minimal essential medium (α -MEM) containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). Bone marrow stromal cells were isolated from 4-week old mice as described (37). The cells were cultured to 80–90% confluence prior to use in experiments. Twenty-four hours prior to treatment, cells were incubated in the presence of serum-free α -MEM medium containing 0.1% bovine serum albumin (BSA) and antibiotics. THs (T3 and T4) stocks were prepared in 5mM NaOH and diluted in α -MEM prior to adding to cell cultures.

Transfection and transduction in osteoblasts

For knockdown studies, MC3T3-E1 cells were transiently transfected by electroporation as reported previously (36,38). Briefly, MC3T3-E1 cells (1.5×10^6) were resuspended in 100 μl of fibroblast nucleofector buffer (Amaxa, Gaithersburg, MD) containing 1.25 μg TR 1/2 siRNA or 1.25 μg negative control siRNA (Santa Cruz). The cells were then transferred into a 2-mm gap width electroporation cuvette, and electroporated at 165 V for 15 milliseconds, using a Gene Pulser (Bio-Rad, Hercules, CA). After electroporation, the cells were plated at high density (150,000 cells/ cm^2) in prewarmed, α -MEM growth medium in a 6-well plate, and cultured in a humidified 37 $^\circ\text{C}$ incubator with 5% CO_2 , 95% air until further treatment. For overexpression studies, MC3T3-E1 cells were transduced with adenoviral particles expressing TR 1, TR 2 or β -Gal control as described (29).

RNA extraction and real-time quantitative polymerase chain reaction

RNA was extracted from MC3T3-E1 cells, primary calvarial osteoblasts, primary bone marrow stromal (BMS) cells or the mouse tissues as described previously (36,39). An aliquot of RNA (2 μg) was reverse-transcribed into cDNA in 20 μl volume of reaction by oligo(dT)_{12–18} primer. Real-time PCR contained 0.5 μl template cDNA, 1 \times SYBR GREEN master mix (Qiagen), and 100 nM of specific forward and reverse primers in a 25 μl reaction volume. Primers used for real-time PCR are listed in Table 1.

Luciferase reporter and ALP activity assays

The pTAL-luc-IGF-I and control reporter constructs were transfected into MC3T3-E1 cells with lipofectamine (Invitrogen). After a 48-hour treatment with TH (10 ng/ml T3) or vehicle, the transfected cells were lysed for luciferase assay as described (36). Alkaline phosphatase (ALP) activity in MC3T3-E1 cells treated with TH or vehicle in serum-free medium for 3 days was measured as previously reported (38).

Electrophoretic mobility shift assay

Nuclear extracts from MC3T3-E1 cells were prepared as described previously (36,39). Double-stranded oligonucleotides were labeled as described previously (36). Nuclear extracts (4 µg) from MC3T3-E1 cells were incubated in a binding buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 3 mM DTT, 10 % glycerol, 0.05% NP-40, 0.1 mM ZnCl₂, 50 µg/ml poly dI-dC, and 20 fmole of labeled DNA probe. Excess unlabeled DNA competitors were added into the reaction 5 minutes before addition of the radiolabeled probe. The reactions were incubated at room temperature for 20 minutes, and then antibody against TR or control IgG was added to the reactions. The reactions were incubated at room temperature for another 20 minutes, and analyzed on 5% non-denaturing polyacrylamide gels in 1 × TBE buffer (50 mM Tris-borate-EDTA, pH 8.0). Gels were dried and visualized by autoradiography.

Mouse Metatarsal Bone Culture

Metatarsal bones were surgically isolated from 2-day old C57BL/6 mice and were incubated in serum-free MEM containing 0.5% BSA, 50 µg/ml ascorbic acid, 1 mM β-glycerol phosphate, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in humidified air with 5% CO₂ as reported (40). T3 (10 ng/ml) or the same volume of vehicle control was added to medium 1 day later, and metatarsals were cultured for 10 days. Imaging was performed at day 10 under a microscope attached to a Nikon camera. The length of metatarsals was measured using a computer with Amira software (Mercury Computer Systems, Inc., Chelmsford, MA).

Statistical Analysis

Data were analyzed by Student's *t*-test or ANOVA as appropriate. One- and two-way ANOVA tests were performed using STATISTICA software (Statsoft, Tulsa, OK).

RESULTS

TH is an important regulator of IGF-I expression and bone acquisition during the prepubertal growth period

To determine if TH is a major regulator of IGF-I production during the prepubertal growth period, we first measured the postnatal changes in serum levels of total T3 and IGF-I in C57BL/6J mice. As seen in Figure 1A, serum levels of IGF-I increased almost three-fold in mice during the prepubertal growth period (i.e. first three weeks after birth). This increase in IGF-I was preceded by changes in serum T3 levels, which increased two-fold between days 7 and 14. The serum levels of T3 correlated with serum IGF-I levels ($r = 0.82$; Figure 1B).

To determine a cause and effect relationship between changes in serum T3 and IGF-I levels, hypothyroidism was induced in newborn mice by treating pregnant mice with 0.05% methimazole in drinking water. The serum level of total T3 in pups from mothers treated with methimazole was significantly reduced by 32% at 14 days of age compared to untreated control mice (Figure 1C). Consistent with a role for TH in regulating IGF-I production, serum levels of IGF-I were reduced by 36% in the TH-deficient mice compared to control mice, and correlated with serum levels of T3 ($r = 0.76$, $p < 0.05$). Body weight and length were reduced by 25% and 10%, respectively, in TH-deficient mice compared to control mice. Furthermore, femur periosteal circumference (PC) and BMD were reduced by 12% and 20%, respectively. IGF-I mRNA levels, determined by real-time RT-PCR, was reduced by 33% ($n = 7$, $P < 0.01$) in the femurs in the hypothyroid mice as compared to control mice.

To obtain further evidence that the increase in IGF-I production during prepubertal growth is dependent on TH, we used two additional mouse models, *Duox2*^{-/-} and *Tshr*^{-/-} which exhibit hypothyroidism (30,41). A missense mutation in the *Duox2* gene results in a 90% reduction in serum T4 (30). Serum levels of IGF-I, femur BMC and BMD were normal in *Duox2*^{-/-} at 7 days of age (Figure 1D). However, at day 21, IGF-I was reduced by 53% in *Duox2*^{-/-} mice. Femur BMC and BMD were decreased by 46% and 25%, respectively, in *Duox2*^{-/-} mice at day 21. A similar phenotype was identified in *Tshr*^{-/-} mice (Figure 1E).

If the reductions in serum IGF-I and bone growth were a direct consequence of a lack of increase in TH production during the prepubertal growth period, it should be possible to rescue these deficiencies by giving TH during the time when TH levels increase. Thus, *Tshr*^{-/-} mice were treated with a combination of 1 µg T3 and 10 µg of T4 per day (42) from days 5 to 14 after birth or with the same volume of 5 mM NaOH vehicle. *Tshr*^{-/+} heterozygous mice that are euthyroid (43) were treated with NaOH vehicle and used as controls. As expected, *Tshr*^{-/-} mice treated with vehicle exhibited significant reductions in both BMC and BMD of total body, femur and vertebra (Figure 1F). T3/T4 treatment rescued these deficits in bone acquisition (Figure 1F). In vehicle treated *Tshr*^{-/-} mice, the serum IGF-I level was reduced by 50% and associated with reduced IGF-I expression in the liver and bone (Figure 1G). TH treatment during prepubertal growth increased IGF-I expression in both liver and bone and normalized the serum IGF-I level.

TH regulates bone and fat phenotypes independent of GH during the prepubertal growth period

If TH is a major regulator of the prepubertal rise in IGF-I expression and bone acquisition, we then should expect a greater deficit in bone mass in TH deficient mice compared to GH deficient mice. We, therefore, examined the skeletal phenotypes of mice deficient in GH, TH or both. Figure 2A shows that deficits in body weight and length were greater in TH deficient mice compared to GH deficient mice. TH or GH deficiency increased the percentage of fat which was further increased by the combined deficiency of GH and TH. Although increased body fat in the hypothyroid animals is anticipated based on the well established effects of TH to accelerate resting energy expenditure, published data from individuals transitioning from hypothyroid to hyperthyroidism and vice versa, as well as from hypothyroid rats placed on a high fat diet failed to establish such a connection (27). The deficits in total body, femur and tibia BMD were significantly greater in the TH deficient mice compared to GH deficient mice (Figure 2B). Surprisingly, *Tshr*^{-/-}; *Ghrhr*^{lit/lit} double-mutant mice that are deficient in both TH and GH did not exhibit a greater BMD deficit than the mice with TH deficiency alone at day 21. We next addressed the question of whether the skeletal deficit in double-mutant mice can be rescued by TH and/or GH treatment between days 5 to 14. Treatment with T3/T4 or GH alone for 10 days significantly decreased body weight as well as length in the double mutant mice (Figure 2C). Combined treatment with GH and T3/T4 did not significantly influence either the body weight or length in double mutant mice. However, treatment of TH deficient *Tshr*^{-/-}; *Ghrhr*^{lit/+} mice with T3/T4 increased both body weight and length. TH treatment rescued the body fat phenotype in the TH deficient mice, and treatment of *Tshr*^{-/-}; *Ghrhr*^{lit/lit} mice with either TH or GH alone, or both also rescued the body fat phenotype. Surprisingly, neither TH nor GH treatment rescued skeletal deficits in the double-mutant mice (Figures 2D and E). TH treatment of double mutant mice decreased total body BMD but had no effect on femur or tibia BMD. GH treatment alone did not influence total body, femur or tibia BMD in the double mutant mice either. However, combined GH and TH treatment during days 5–14 increased total body, femur and tibia BMD in double-mutant mice. In addition, TH administration alone rescued deficits in total body, femur and tibia BMD in TH deficient *Tshr*^{-/-}; *Ghrhr*^{lit/+} mice.

Micro-CT analysis demonstrated a decrease in trabecular bone volume in TH deficient mice, which was rescued by TH replacement (Figures 2F and G). Neither TH nor GH treatment alone was sufficient to rescue the decreased trabecular bone volume in *Tshr*^{-/-}; *Ghrhr*^{lit/lit} double mutant mice. However, treatment of double-mutant mice with both GH and TH restored trabecular bone volume. TH replacement increased trabecular thickness in TH deficient mice while the combined treatment of TH and GH increased trabecular thickness and decreased trabecular separation in *Tshr*^{-/-}; *Ghrhr*^{lit/lit} double mutant mice.

Consistent with the bone phenotypic changes, TH treatment increased serum IGF-I levels in the TH deficient mice which was accompanied by increases in expression of both IGF-I and acid-labile subunit (ALS) in the liver (Figure 2H). Serum IGF-I level was decreased by 84% ($P < 0.001$) in *Tshr*^{-/-}; *Ghrhr*^{lit/lit} mice compared to *Tshr*^{+/-}; *Ghrhr*^{lit/+} control mice. Treatment of double mutant mice with T3/T4, GH or T3/T4 + GH resulted in a 20–40% decrease in serum IGF-I compared to vehicle treatment. Accordingly, IGF-I and ALS mRNA levels were reduced in the double mutant mice treated with T3/T4, GH or T3/T4 + GH compared to vehicle treatment.

TH directly regulates IGF-I transcription predominantly via TR α in osteoblasts

To determine whether TH regulates IGF-I expression directly, we treated MC3T3-E1 cells in serum-free conditions with T3 and demonstrated a dose- and time-dependent effect of T3 on IGF-I mRNA levels (Figures 3A and 3B). IGF-I expression increased by 8-fold after 24 hrs of treatment with 10 ng/ml of T3. IGF-I protein in serum-free conditioned medium was increased by 5-fold in both MC3T3-E1 and primary osteoblasts after T3 treatment (Figure 3C). Furthermore, the half-life of IGF-I mRNA was similar between T3 and vehicle control (Figure 3D).

To determine which TR isoform is predominantly expressed in primary BMS cells, calvarial osteoblasts and MC3T3-E1 cells, transcript levels of TR 1, TR 2, TR 1, TR 2 and TR 1 were determined by RT-PCR. TR 1, TR 2 and TR 1 were expressed in all 3 cell types but expression of TR 1, TR 2 and TR 2 mRNAs was undetectable (Figures 4A & B). TR 1 expression was 32-fold and 6-fold greater than TR 1 and TR 2, respectively in MC3T3-E1 cells. To evaluate the role of TR 1 in mediating TH effects on IGF-I expression, we transfected MC3T3-E1 cells with a siRNA that targets both TR 1 and TR 2 or control siRNA. MC3T3 cells transfected with TR 1 siRNA led to a 70% and 90% reduction in expression of TR 1 and TR 2, respectively (data not shown). By contrast, expression of TR 1 was unaffected in TR 2 siRNA treated cells (1.05 ± 0.2 , $n = 3$, $P = 0.78$). TR 1 siRNA treatment led to a greater than 50% inhibition of the TH-induced increase in IGF-I expression in MC3T3-E1 osteoblasts (Figure 4C). Overexpression of TR 1 but not TR 2 resulted in upregulation of IGF-I expression by T3 stimulation (Figure 4D). Osteocalcin mRNA expression was increased in TR 1 overexpressed cells as compared to the control cells that expressed -gal (54.6 ± 3.4 vs 40.2 ± 3.7 fold, $P < 0.05$). There was no significant change in osteocalcin expression in the MC3T3-E1 cells overexpressing TR 2 (42.1 ± 5.2 vs 40.2 ± 3.7 fold, $P = 0.64$). While neither TR 1 nor TR 2 overexpression decreased basal IGF-I expression, TR 2 overexpression resulted in a 40% decrease ($P < 0.01$) in basal osteocalcin expression.

Because of the rapid effect of T3 on IGF-I expression, we predicted a direct effect of TH on IGF-I gene transcription. We identified a putative TRE in intron 1 of the mouse IGF-I gene located between nucleotides 2113 to 2152 downstream of the transcription start site (Figure 5A). To determine whether TR 1 present in mouse osteoblasts binds to this putative TRE, EMSA was carried out. As shown in figure 5B, nuclear extracts from MC3T3-E1 cells bound specifically to the putative TRE. The formation of DNA-protein complex was sequence specific since the presence of a 200-fold excess of unlabeled IGF-I TRE or a

consensus TRE (44), but not an unlabeled SP1 binding site was able to compete for the binding of the nuclear extract to the labeled IGF-I TRE. Furthermore, the protein-DNA complex was disrupted by a TR antibody but not by control IgG (Figures 5B, lane 6 & 7). To determine whether the IGF-1 TRE mediates TH effects on IGF-I transcription, a 155 bp IGF-I intron 1 fragment containing the putative TRE was inserted upstream of the TK minimal promoter in pTAL-Luc to generate a pTAL-Luc-IGF-I reporter construct. MC3T3-E1 cells were transiently transfected with reporter plasmid, and treated with 10 ng/ml T3 or vehicle for 48 hours prior to luciferase assay. As shown in 5C, T3 treatment increased luciferase activity by 2.3-fold.

TR α and TR β differentially regulate expression of IGF system components in mice

To determine the role of TRs in regulating IGF-I expression *in vivo*, we measured serum levels of IGF-I in homozygous and heterozygous mice lacking functional TR or TR and their corresponding WT littermate controls. Serum IGF-I was increased in homozygous TR KO mice compared to both WT and heterozygous littermates (Figure 6A). By contrast, homozygous mutant mice lacking both TR 1 and 2 showed a 29% decrease ($P < 0.05$) in serum IGF-I compared to heterozygous and WT mice. Accordingly, IGF-I mRNA expression was elevated in TH target tissues including the bone and the cartilage in homozygous TR KO mice (Figure 6B). By contrast, IGF-I expression was decreased in the liver but not in other tissues of TR KO mice (Figure 6C). Furthermore, ALS and IGFBP-3 expressions were increased in the liver in homozygous TR KO mice but decreased in TR KO mice (Figures 6D and E). IGFBP-5 mRNA expression was unaffected by deletion of either TR or TR (Figure 6F).

TH biological effects on bone cells is in part dependent on local IGF-I

We next addressed if TH effects on bone depend on local IGF-I production. We evaluated the effects of T3 on ALP activity in MC3T3-E1 cells after neutralizing IGF activity with an excess of inhibitory IGFBP4. TH-induction of ALP activity was inhibited by IGFBP4 (Figure 7A). To examine whether local IGF-I mediates TH effects on bone growth, metatarsals were isolated from 3-day old IGF-I conditional KO mice, and incubated in serum-free conditions in the absence or presence of TH for 10 days. Total and mineralized lengths were measured from digital images of the metatarsals (Figure 7B). Ten days of TH treatment resulted in a 25% increase in mineralized length adjusted for total length whereas only a 10% increase was observed in the mice lacking IGF-I in osteoblasts (Figure 7C). Consistent with these data, mRNA levels of transcription factors (Runx2, Osx and Dlx5) and osteocalcin were increased to a greater extent by 10 days of TH treatment compared to vehicle in the metatarsals from WT mice compared to the metatarsals from IGF-I conditional KO mice (Figure 7D). By contrast, TH treatment caused a similar increase in the mRNA levels of TRAP, a marker of bone resorption, in the metatarsals-derived from WT and IGF-I conditional KO mice.

DISCUSSION

The importance of IGF-I in skeletal development is well established since peak bone mass is considerably reduced as a consequence of deficiency in IGF-I action in both humans and experimental animals (6–9,14,21). In terms of mechanisms for IGF-I regulation, experimental data from genetic mouse models lacking IGF-I or GH revealed that GH-independent mechanisms predominantly contribute to bone acquisition during prepubertal growth while GH-dependent mechanisms contribute predominantly to bone acquisition during both pubertal and postpubertal growth periods (7). There are a number of potential explanations for the limited contribution of GH during the prepubertal period compared to the pubertal growth period. First, serum levels of GH increase several fold during the

pubertal growth spurt. Thus, lower levels of GH during prepubertal growth may contribute to reduced GH effects on target tissues. Second, studies have shown that the relatively low contribution could be due to GH insensitivity during the prepubertal growth period. In this regard, we found that the gain in bone in response to GH therapy is several fold greater when GH was administered during the pubertal growth period compared to the prepubertal growth period, thus suggesting that GH effect on bone acquisition is growth-period dependent (12). In terms of the mechanism for the reduced GH sensitivity in target tissues during the prepubertal growth period, studies by Waxman et al. (45) have shown that certain liver factors necessary for mediating GH effects are absent in prepubertal rats. Third, a cooperative crosstalk between IGF-I and estrogen receptor signaling has been well documented (46) which could contribute to greater GH action during pubertal and postpubertal growth periods.

In this study, we demonstrate that TH-dependent mechanisms contribute to prepubertal regulation of IGF-I action and bone acquisition. Accordingly, the magnitude of the skeletal deficit is approximately 3 times greater in TH-deficient mice compared to GH-deficient mice at the end of prepubertal growth period. The findings that mice deficient in both GH and TH did not exhibit greater BMD deficits than mice deficient in TH alone indicate that TH and GH may be acting via a common pathway to regulate bone density. The skeletal phenotype data from genetic mouse models deficient in TH and/or GH action have provided unequivocal evidence that there is a critical time period prior to puberty, when the effects of GH are small and TH plays a key role in the regulation of skeletal growth.

The findings that treatment of TH deficient mice with daily administration of T3/T4 during the 10 day period (5 to 14 days of age) when serum T3 levels are elevated rescues deficiencies in body weight, bone length as well as bone density suggest that the deficits in both somatic and bone growth in TH deficient mice were a consequence of the lack of an increase in T3 production during the prepubertal growth period. The findings from TR 1 knock-in mutant mice also provide evidence for a direct T3 effect on bone (25,47). Neither TH nor GH treatment alone rescued the skeletal deficit in the double-mutant mice lacking both GH and TH. While combined treatment with both GH and TH rescued the bone density deficit in double- mutant mice, it did not completely rescue body weight or bone length. By contrast, GH or TH treatment alone reduced body fat in double-mutant mice. In terms of the question why TH alone was ineffective in stimulating bone acquisition in TH/GH double mutant mice, there is now a considerable amount of clinical data that demonstrate normal TH action is necessary for the optimal response to both endogenous and recombinant hGH substitution (48). Thus, TH may sensitize GH effects in bone. If all of the TH effect is dependent on GH, we hypothesized that GH-deficiency would result in a similar reduction in bone density at the end of the prepubertal growth period. However, the current studies do not support this hypothesis. Thus, TH exerts direct GH-independent effects on bone in addition to sensitizing skeletal responses to GH.

A key finding is that TH regulates IGF-I gene transcription via a TRE located in intron 1 of the IGF-I gene. Consistent with direct effects of TH on IGF-I gene expression, TH treatment during the prepubertal growth period rescued the deficit in IGF-I mRNA level in both liver and bone. IGF-I in serum is short lived unless it is bound to the IGFBP-3/ALS complex. Our findings also provide the first experimental evidence that TH increases ALS expression in the liver, thus suggesting that the rise in serum levels of IGF-I during the prepubertal growth period may be mediated via both a TH-dependent increase in IGF-I as well as ALS expression. Surprisingly, combined treatment with GH and TH in the double-mutant mice for two weeks did not increase either IGF-I nor ALS expression in the liver. Accordingly, serum IGF-I levels remained unchanged in response to GH+TH treatment in the double-

mutant mice. These data suggest that the mechanisms that mediate effects of TH and GH on target genes are complex.

Our *in vitro* findings from experiments evaluating the consequence of TR knockdown as well as overexpression on IGF-I mRNA levels in MC3T3-E1 osteoblasts, and the findings from EMSA experiments that TR¹ bound to a TRE in the IGF-I gene, indicate that ligand bound TR¹ is an activator of IGF-I gene transcription. While TR¹ may be the predominant receptor involved in mediating TH effects in skeletal cells because it is expressed at higher levels than TR², these studies do not exclude a similar role for TR² in target tissues such as the liver where it is highly expressed. If TR¹ is a positive regulator of IGF-I expression, we predict that mice lacking TR¹ would exhibit reduced serum IGF-I levels compared to control mice. However, TR^{0/0} mice have elevated serum IGF-I levels. Since TR^{0/0} KO mice are euthyroid (31), this unexpected finding may result from the systemic consequences of the global deletion of TR^{0/0}. Furthermore, results from *in vitro* studies on TH effects on IGF-I gene expression in serum-free conditions may not recapitulate the effects of TH *in vivo*. Alternatively, in the absence of TR¹, it is possible that the TRE in intron 1 of the IGF-1 gene may be occupied by other transcription factors such as AP1 and SF-1 that result in enhanced IGF-1 transcription (49,50).

In an earlier study, O'Shea et al. (47) reported diminished expression of GH receptor, IGF-I and IGF-I receptor in the growth plates of heterozygous TR^{1PV} mutant mice carrying the PV mutation targeted to TR¹. The mutant TR^{1PV} protein acts as a potent dominant negative receptor that interferes with transcriptional activities of WT TR¹ and TR² receptors. Thus, the findings in TR^{1PV} mice compared to TR^{0/0} KO mice may result from additional effects of the PV mutant protein that are similar to the effect of hypothyroidism in which unliganded TR¹ acts as a repressor of target gene expression.

By contrast, circulating IGF-1 and hepatic IGF-1 expression were decreased in TR^{0/0} KO mice. TR^{0/0} KO mice have chronically elevated circulating T4 and T3 levels due to disruption of the pituitary thyroid negative feedback axis (32,51). The lack of TR¹ expression in the liver in TR^{0/0} KO mice results in reduced hepatic IGF-I expression despite elevated systemic TH levels because the liver, in contrast to the bone, is a TR¹ responsive target tissue. Thus, in the skeleton, elevated TH in TR^{0/0} KO mice increased IGF-I expression via TR¹ transactivation.

The findings that inhibition of IGF-I action only partially reduced TH effects on osteoblast differentiation and the rate of metatarsal bone mineralization indicate that TH acts via other pathways in addition to IGF-I. Consistent with this, it is known that TH influences FGF receptor signaling in bone and interacts with the Wnt/ β -catenin and Ihh/PTHrP signaling pathways to regulate endochondral ossification (52–54). Wang et al. (54) have demonstrated that TH stimulates IGF-I expression in growth plate chondrocytes to induce proliferation. Furthermore, they also have shown that the TH effect on chondrocyte hypertrophy is mediated via activation of Wnt/ β -catenin signaling which is in part initiated by IGF-I signaling. Future studies are needed to determine whether a similar mechanism is involved in mediating TH effects on osteoblast differentiation.

The limitations of this study include the inability of the methods used to differentiate between different bone (cortical/trabecular) and fat (white/brown) compartments, the short window of time used to study the growth phenotype and the single dose of T3/T4 used for rescue experiments. Despite these limitations, our studies using different mouse mutants show that TH acting via IGF-I-dependent and -independent mechanisms plays a more critical role than GH in the regulation of skeletal growth during the prepubertal growth period.

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Author's roles: W.X., K.G., and S.M. designed experiments and performed data analysis and interpretation. C.K. and J.E.W. assisted in the skeletal phenotype measurements. CL assisted in the TH receptor work. AG and AW assisted in the gene expression work involving TR and TR KO mice. L.R.D. contributed to data involving Duox2 KO mice. W.X. and S.M. wrote the manuscript with assistance from G.R.W. All authors provided critical revisions for the manuscript. S.M. conceived and oversaw the entire the project.

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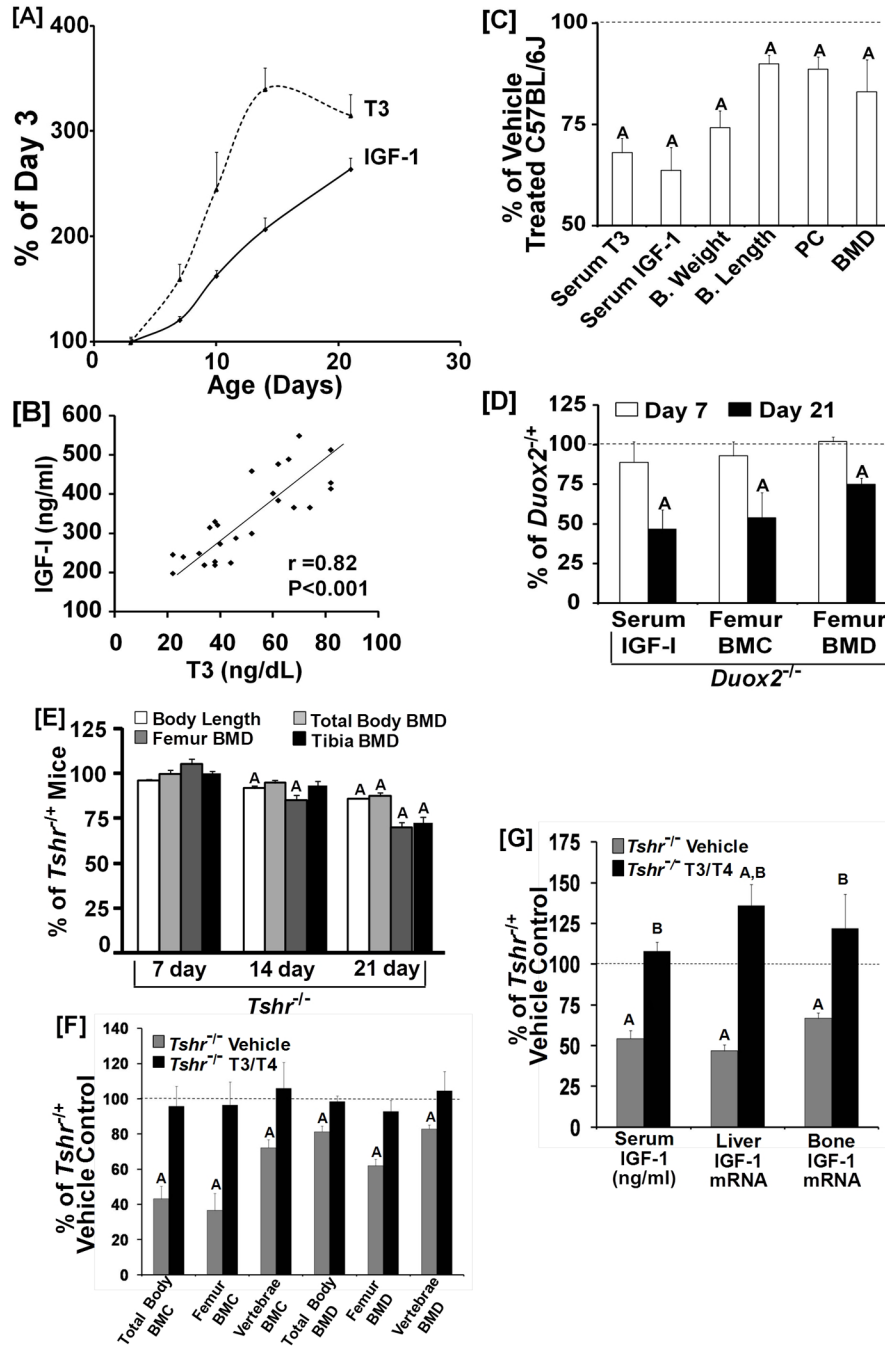


Figure 1. TH is essential for the prepubertal increase in serum IGF-I level and bone mass
 [A]: Postnatal changes in serum levels of total T3 and IGF-I in C57BL/6J mice. Values are percentage of day 3 and are mean \pm SEM (n = 7). IGF-I and T3 levels at day 3 were 88 ng/ml and 20 ng/dl, respectively. A = P < 0.05 as compared to day 3. [B]: Correlation between serum levels of total T3 and IGF-I during prepubertal growth in C57BL/6J mice. [C]: Serum and skeletal parameters in pups from methimazole treated experimental mice at day 14 of age. Values are percentage of control mice and are mean \pm SEM (N = 7 to 16). A = P < 0.05 vs. control. B = Body; PC = periosteal circumference. [D]: Serum levels of IGF-I, femur BMC and femur BMD in *Duox2* mutant and control mice at 7 and 21 days of age. Values

are % of day 7 and 21 control mice (Mean \pm SEM, n = 5 to 12). A = P < 0.01 vs. control mice. **[E]**: Body length and BMD in TH-deficient *Tshr*^{-/-} and *Tshr*^{+/-} control mice at 7, 14 and 21 days of age. Values are percentage of day 7 of control mice (Mean \pm SEM, n = 8). A = P < 0.01 vs corresponding control mice. **[F-G]**: Effects of T3/T4 treatment (days 5–14) on skeletal **(F)** and IGF-I **(G)** phenotype in TH-deficient *Tshr*^{-/-} mice. At day 21, mice were euthanized and used for end point analyses. Values are % of H/+ vehicle (5 mM NaOH) treated control mice (Mean \pm SEM, n = 8. A = P < 0.01 vs. control mice, B = P < 0.01 vs. corresponding vehicle treated mice.

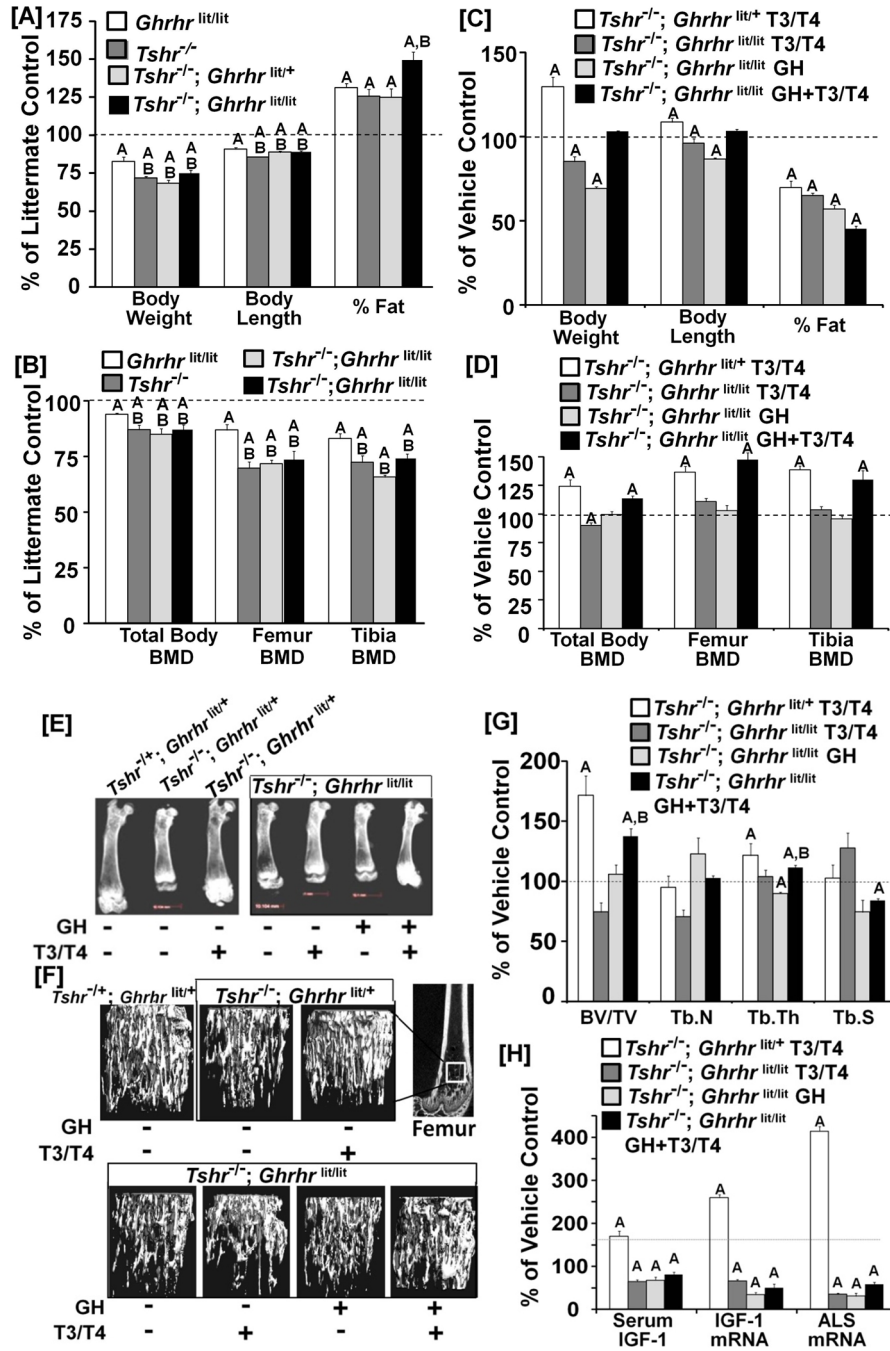


Figure 2. TH regulates bone and fat phenotypes independent of GH during the prepubertal growth period
[A-B]: Body weight, length and percentage of fat (A) and total body, femur and tibia BMD (B) in *Ghrhr*^{lit/lit}, *Tshr*^{-/-} and *Tshr*^{-/-}; *Ghrhr*^{lit/+}, or *Tshr*^{-/-}; *Ghrhr*^{lit/lit} mice. Values are percentage of littermate control (*Ghrhr*^{lit/+}; *Tshr*^{-/+}). A = P < 0.01 vs. control littermate mice (n = 10 to 16/per group). A = P < 0.05 vs WT littermate control mice. B = < 0.05 vs *Ghrhr*^{lit/lit} mice. **[C-D]:** Treatment effects of T3/T4, GH and GH + T3/T4 on body weight, length and percentage of fat (C) and total body, femur and tibia BMD (D) in *Tshr*^{-/-}; *Ghrhr*^{lit/lit} mice. *Tshr*^{-/-}; *Ghrhr*^{lit/+} mice treated with T3/T4 were used as positive control. Values are percentage of vehicle treated mice of the same genotype. A = P < 0.01 vs. vehicle

treated mice of the same genotype (n = 7 to 10 per group). *Tshr*^{-/-}; *Ghrhr*^{lit/lit} and *Tshr*^{-/-}; *Ghrhr*^{lit/+} mice were treated with T3/T4 or vehicle from day 5 to day 14 after birth. For GH treatment, 4mg/kg body weight dose of GH was equally divided into two doses and administered once in the morning and once in the evening daily. **[E]**: Representative X-ray images of the femurs isolated from the mice in C. **[F]** Representative μ -CT images of distal metaphysis of femurs from the mice in C. **[G]** Effect of TH and/or GH treatment on bone volume (BV)/total volume (TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular spacing (Tb.S) of distal metaphysis of femur in mice in C, measured by μ -CT. A = P < 0.01 vs vehicle treated control mice of the same genotype, B = P < 0.01 vs. GH treated *Tshr*^{-/-}; *Ghrhr*^{lit/lit} mice. **[H]**: Effects of TH and/or GH on serum level of IGF-I, IGF-I mRNA and ALS mRNA in the livers isolated from the mice in C. A = P < 0.01 vs. vehicle treated mice of the same genotype (n = 7 to 10 per group).

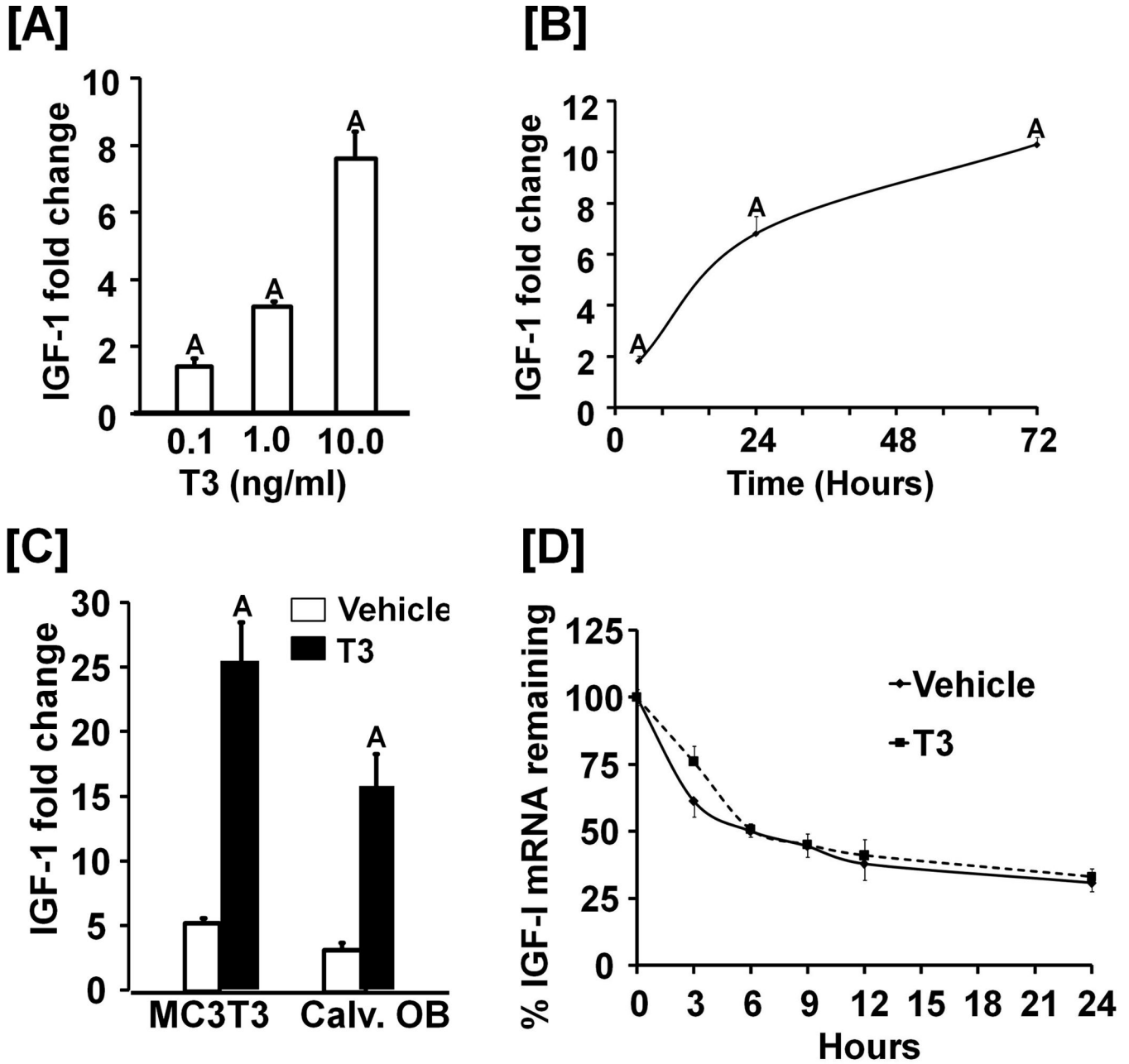


Figure 3. T3 stimulates IGF-1 expression in osteoblasts

[A-B]: Dose (A) and time (B) –dependent effects of T3 on IGF-I expression in MC3T3-E1 cells. For the dose response experiment, cells were treated with 0.1–10 ng/ml of T3 for 24 hours. For time course studies, cells were treated with 10 ng/ml of T3 for 24 hours. Values are fold-change over the expression level of vehicle treated cells and expressed as the mean \pm SEM (n = 3). A = P < 0.01 vs. vehicle control. [C]: T3 stimulates IGF-I protein expression. MC3T3-E1 cells or primary osteoblasts (Calv. OB) were treated with 10 ng/ml of T3 for 72 hours. Conditioned medium was collected for measurement of secreted IGF-I by RIA. Values are the mean \pm SEM (n = 3). A = P < 0.01 vs. vehicle control. [D] Effect of T3 treatment on IGF-I mRNA stability. MC3T3-E1 cells were treated with 10 ng/ml T3 or vehicle for 24 hrs prior to the addition of actinomycin (10 μ g/ml). Cells were harvested for

RNA extraction and real time RT-PCR at the time points indicated. Data are expressed as mean \pm SEM from 3 replicates.

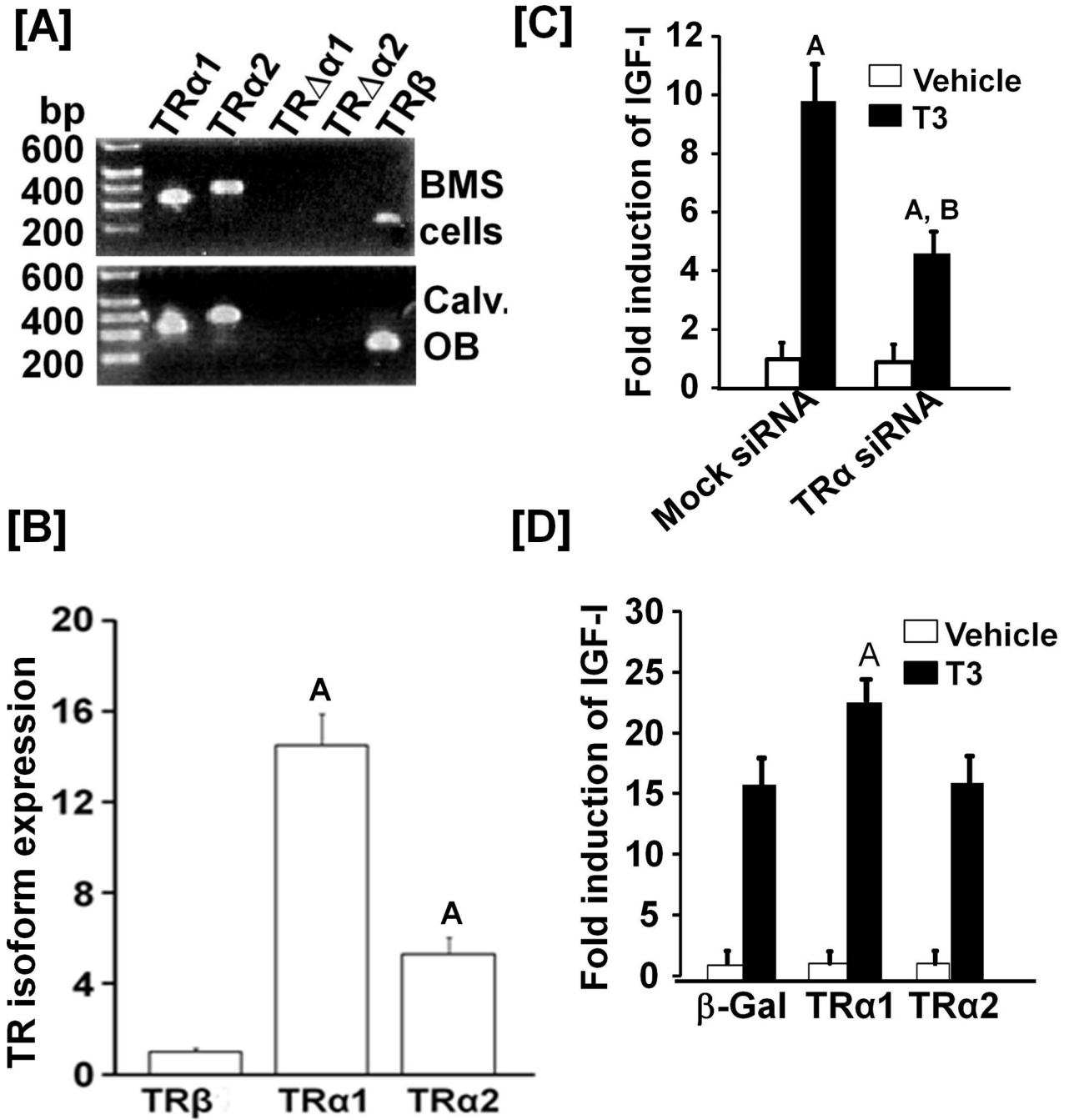


Figure 4. TR α 1 is predominantly expressed in bone cells and mediates T3 induction of IGF-I expression

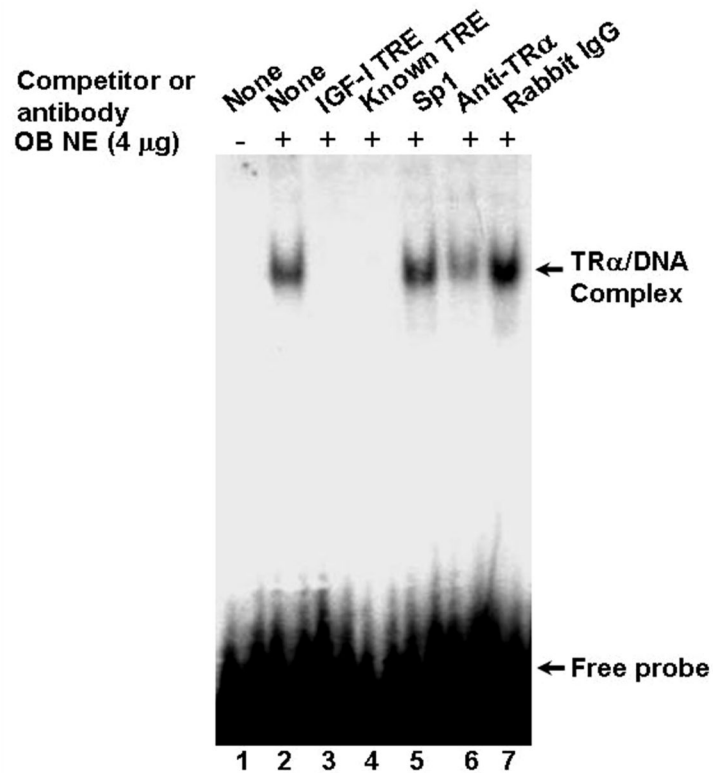
[A]: Expression levels of various isoforms of TH receptors in osteoblasts. RNA extracted from bone marrow stromal (BMS) cells and calvarial osteoblasts (Calv. OB) were reverse transcribed for RT-PCR. PCR products were visualized on an agarose gel. **[B]:** Quantitative analyses of TH receptor (TR) expression in primary osteoblasts by real time RT-PCR. Values are expressed as percentage of TR α 1 and mean \pm SEM (n = 3). A = P < 0.01 vs. TR α 1. **[C]:** Effect of knockdown of TR α 1 expression by siRNA on the TH-induced increase in IGF-I expression. Twenty-four hours after transfection of MC3T3-E1 cells with siRNA specific to TR α 1/2 or control siRNA, the cells were treated with 10 ng/ml T3 or vehicle for

another 24 hrs prior to RNA extraction and real-time PCR. Values are the mean \pm SEM (n = 3). A = P < 0.01 vs. vehicle; B = P < 0.01 vs corresponding control siRNA treatment. **[D]**: Effect of overexpression of TR 1, TR 2 and -gal on the TH-induced increase in IGF-I expression. Twenty-four hours after transduction of MC3T3-E1 cells with adenoviral particles expressing -gal, TR 1 or TR 2, cells were treated with T3 (10 ng/ml) or vehicle. After 24 hrs of treatment, RNA was extracted for real time RT-PCR. Values are the mean \pm SEM (n = 3).

[A] Partial Intron Sequence of IGF-I gene:

5'GAGAAACCCTCTTCCCAAGGCAGCCATAAACTTGAACATTTGCT
 TCTGACCT^{TRE}cccaTGACTTCCAGTAGAGGAGCTGGCCAGAGAGAAGT
 CTGCTGCCTGCCCTGTCTGATGGCAGGCCACTGGCCCCCTCCCCCA
 GAGGGTCTAAATCTT3'

[B]



[C]

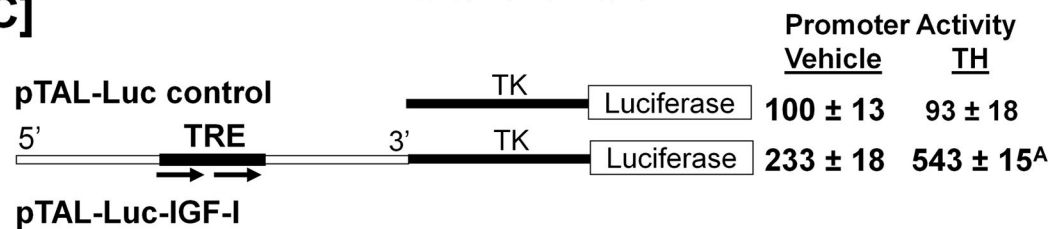


Figure 5. T3 directly regulates IGF-I transcription

[A]: DNA sequence of intron 1 of the mouse IGF-I gene contains putative TRE. [B]: TR binds to the TRE in the mouse IGF-I gene. EMSA was conducted using 4 μ g of nuclear extract (NE) with a ³²P-labeled TRE probe. The sequences of the oligonucleotides are as follows: IGF-I intron TRE: 5'TACTGGAAGTCATTGGGAGGTCAGA; Consensus TRE: 5'AGCTTCAGGTCACAGGAGGTCAGA; Sp1: 5'ATTCGATCGGGCGGGGCGAGC. [C]: TH stimulates luciferase activity of pTAL-IGF-I reporter. A portion of the IGF-I intron containing TRE (top panel) was inserted in front of TK minimal promoter of the pTAL-Luc vector to generate the pTAL-IGF-I reporter. MC3T3-E1 cells were transfected with the

reporter constructs, and then treated with 10 ng/ml T3 or vehicle. Luciferase activity was analyzed 48 hrs after treatment and expressed as percentage of the luciferase activity from the cells transfected with pTAL-Luc and treated with vehicle. $A = P < 0.01$ vs. vehicle treated pTAL-IGF-I.

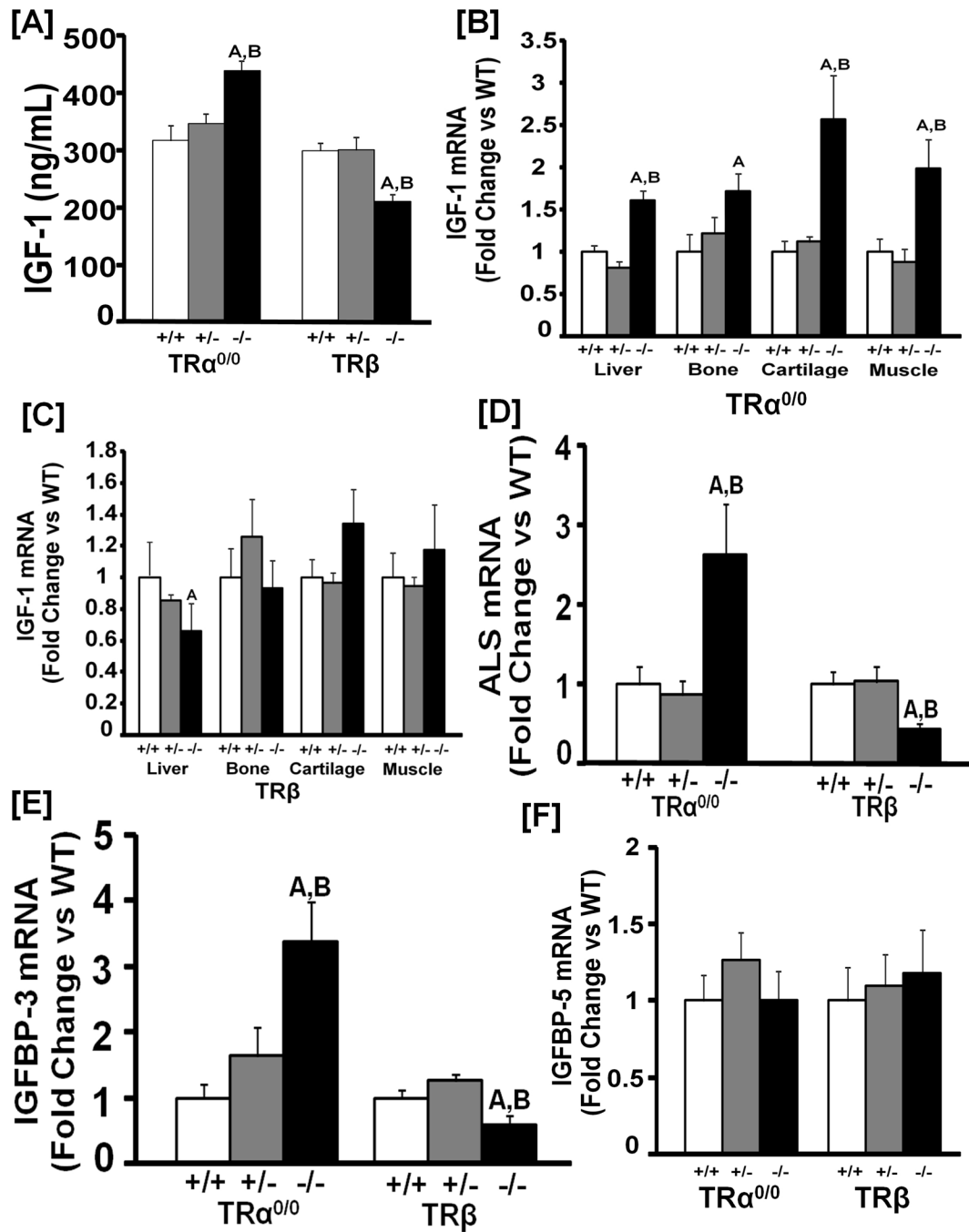


Figure 6. Targeted disruption of $TR\alpha$ and $TR\beta$ differentially modulates IGF-I expression in mice
[A]: Serum IGF-I levels in homozygous and heterozygous $TR\alpha^{0/0}$ and $TR\beta$ mutant mice and corresponding WT littermates at 14 days of age. A = $P < 0.01$ vs WT mice of the same genotype, B = $P < 0.01$ vs heterozygous mice of the same genotype. N = 8–12 mice per genotype. **[B–C]:** IGF-I mRNA levels in the liver, bone, cartilage and muscle of homozygous and heterozygous $TR\alpha^{0/0}$ (**B**) and $TR\beta$ (**C**) mutant mice and corresponding WT littermate control mice at 14 days of age. Values are fold-change of WT control mice. A = $P < 0.01$ vs WT mice of the same genotype, B = $P < 0.01$ vs heterozygous mice of the same genotype. N = 8 mice per group. **[D–F]:** ALS (**D**), IGFBP-3 (**E**) and IGFBP-5 (**F**) mRNA

levels in the liver of homozygous and heterozygous TR^{0/0} and TR mutant mice and corresponding WT littermates at 14 days of age. A = P<0.01 vs WT mice of the same genotype, B = P<0.01 vs heterozygous mice of the same genotype. N = 8–12 mice per genotype.

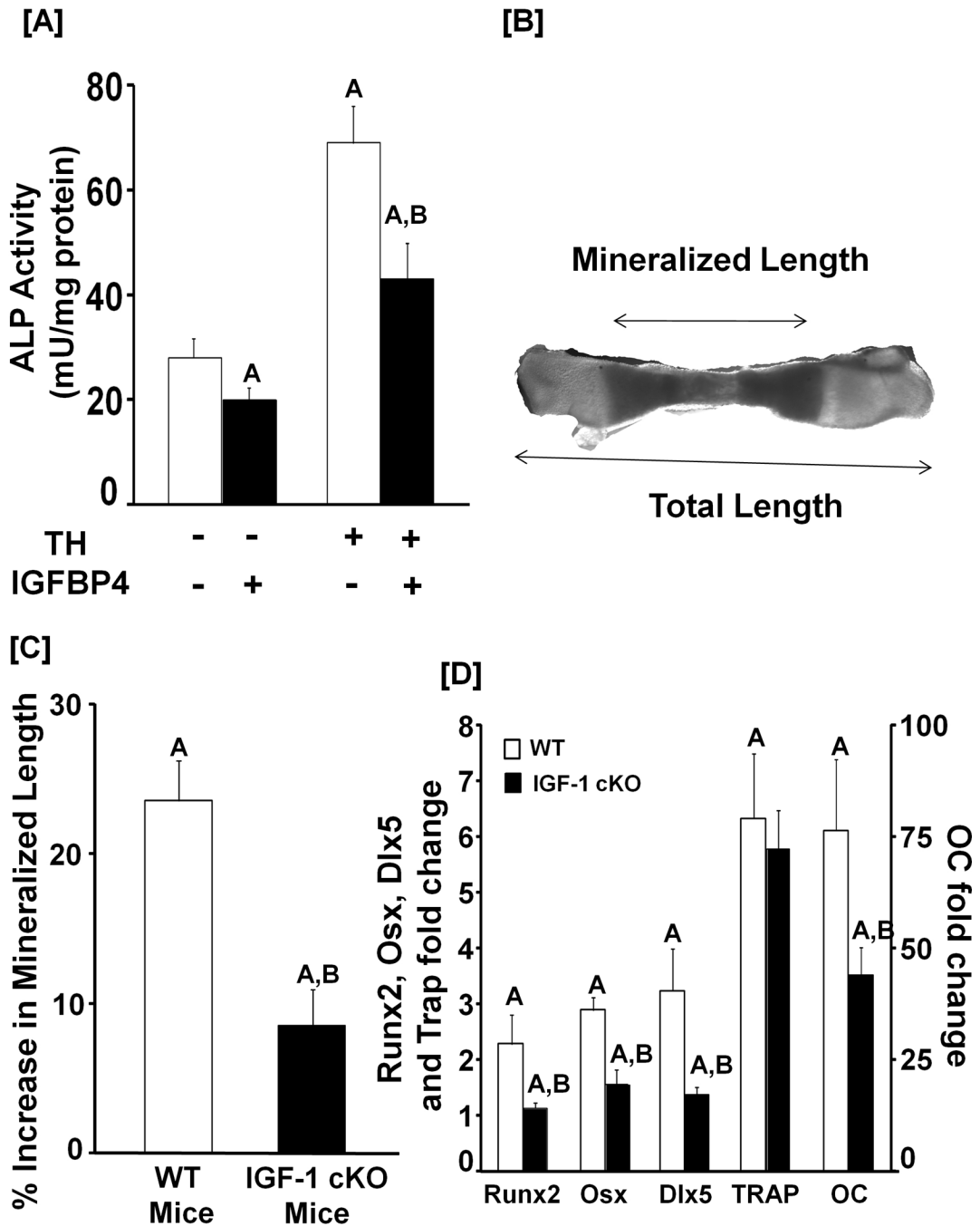


Figure 7. TH biological effects in bone cells is in part mediated via local IGF-I

[A]: Neutralization of IGF-I with inhibitory IGFBP-4 blocks the TH-induced increase in ALP activity. MC3T3-E1 cells were incubated with or without T3 or IGFBP-4. Three days later, cells were lysed for ALP activity determinations and total protein measurements. Values are the Mean \pm SEM (n = 8). A = P < 0.01 vs. vehicle control. B = P < 0.01 vs. T3.

[B]: A microscope image of a mineralized metatarsal bone-derived from a 3-day old WT mouse after a 10 day incubation in DMEM containing 0.5% BSA, 50 μ g/ml ascorbic acid and 1 mM β -glycerol phosphate. **[C]:** Quantitative data of cultured metatarsals-derived from WT and IGF-I conditional knockout (cKO) mice. Metatarsals from 3-day old IGF-I

conditional KO and WT control mice were incubated in serum-free medium containing 0.5% BSA 50 $\mu\text{g/ml}$ ascorbic acid and 1 mM β -glycerol phosphate for 10 days in the presence (10 ng/ml) or absence of T3. The mineralized length of the bone was determined using microscopy. The TH-induced increase in the length of mineralized metatarsals-derived from IGF-I cKO was compared to the metatarsals from the corresponding control WT mice. Mineralized length was adjusted for total length to account for differences in the length of metatarsals for the two genotypes. A = $P < 0.01$ vs. vehicle treated metatarsals. B = $P < 0.01$ vs. T3 treated metatarsals from WT mice. **[D]**: TH-increases the expression of bone specific transcription factors and bone formation markers. RNA was isolated from the cultured metatarsals in C, and used for real time RT-PCR for measurement of expression of transcription factors and bone formation markers as described in the methods. A = $P < 0.01$ vs. vehicle treated metatarsals. B = $P < 0.01$ vs. T3 treated metatarsals from WT mice.

Table 1

Primer sequences for RT real-time PCR

| GENE | FORWARD | REVERSE |
|-------------|-------------------------------|-------------------------------|
| ALS | 5'- GCTCTGTGGCTTGACGGAAAC | 5'- CCAGGAGGTTGTTGCCAAA |
| DLX5 | 5'- AGAAGAGTCCCAAGCATCCGA | 5'- GCCATAAGAAGCAGAGGTAGG |
| IGFBP-3 | 5'- CCAGAACTTCTCCTCCGAGTCTAAG | 5'- CTCAGCACATTGAGGAACTTCAGAT |
| IGFBP-5 | 5'- ATACAACCCAGAACGCCAGCT | 5'- ACCTGGGCTATGCACTTGATG |
| IGF-1 | 5'- GCTCTTCAGTTCGTGTGTGGAC | 5'- CATCTCCAGTCTCCTCAGATC |
| OSTEOCALCIN | 5'- CTCTCTCTGCTCACTCTGCT | 5'- TTTGTAGGCGGTCTTCAAGC |
| OSTERIX | 5'- AGAGGTTCACTCGCTCTGACGA | 5'- TTGCTCAAGTGGTCGCTTCTG |
| PPIA | 5'- CCATGGCAAATGCTGGACCA | 5'- TCCTGGACCCAAAACGCTCC |
| RUNX2 | 5'- AAAGCCAGAGTGGACCCCTTCCA | 5'- ATAGCGTGCTGCCATTTCGAGGT |
| TRAP | 5'- CACTCAGCTGCTCCTGGCTCAA | 5'- CTGCAGGTTGTGGTCATGTCC |
| TR 1 | 5'- CTGCCTTGCGAAGACCAGATC | 5'- CAGCCTGCAGCAGAGCCACTTCCGT |
| TR 2 | 5'- AATGGTGGCTTGGGTGTGGT | 5'- CCTGAACAACATGCATTCCGA |
| TR * | 5'- GGTGCTGGATGACAGCAAGA | 5'- GCATTACGATGGGTGCTTGT |
| TR 1 | 5'- CTCTGTGATCCTGCTGTTCCACAG | 5'- TTTCATGTGGAGGAAGCGGC |
| GC TR 2 | 5'- CTCTGTGATCCTGCTGTTCCACAG | 5'- CCTGAACAACATGCATTCCGA |

* Recognizes both TR 1 and TR 2