

# Genetic confirmation for a central role for TNF $\alpha$ in the direct action of thyroid stimulating hormone on the skeleton

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**Clinical data showing correlations between low thyroid-stimulating hormone (TSH) levels and high bone turnover markers, low bone mineral density, and an increased risk of osteoporosis-related fractures are buttressed by mouse genetic and pharmacological studies identifying a direct action of TSH on the skeleton. Here we show that the skeletal actions of TSH deficiency are mediated, in part, through TNF $\alpha$ . Compound mouse mutants generated by genetically deleting the *Tnfa* gene on a *Tshr*<sup>-/-</sup> (homozygote) or *Tshr*<sup>+/-</sup> (heterozygote) background resulted in full rescue of the osteoporosis, low bone formation, and hyperresorption that accompany TSH deficiency. Studies using ex vivo bone marrow cell cultures showed that TSH inhibits and stimulates TNF $\alpha$  production from macrophages and osteoblasts, respectively. TNF $\alpha$ , in turn, stimulates osteoclastogenesis but also enhances the production in bone marrow of a variant TSH $\beta$ . This locally produced TSH suppresses osteoclast formation in a negative feedback loop. We speculate that TNF $\alpha$  elevations due to low TSH signaling in human hyperthyroidism contribute to the bone loss that has traditionally been attributed solely to high thyroid hormone levels.**

bone metabolism | thyroid disease | bone density

Clinical and biological observations underscore the importance of the direct effects of thyroid-stimulating hormone (TSH) on the skeleton as they relate to the pathogenesis of osteoporosis seen in human hyperthyroidism (1–7). First, we showed that mice lacking the TSH receptor (*Tshr*<sup>-/-</sup>) or expressing a signaling-deficient receptor (*Tshr*<sup>J<sup>hyt</sup>/hyt</sup>) suffer from profound bone loss despite thyroid hormone supplementation (1, 2). Second, when rendered hyperthyroid, *Tshr*<sup>-/-</sup> mice lose more bone than wild-type hyperthyroid mice with a normal TSH axis (3); this suggests that TSH signaling deficiency contributes to the osteoporosis that has hitherto been attributed solely to thyroid hormone excess. Third, the greater bone loss in hyperthyroid *Tshr*<sup>-/-</sup> mice compared with wild-type hyperthyroid mice with undetectable serum Tsh levels is likely due to the osteoprotection exerted by a Tsh $\beta$  variant (Tsh- $\beta$ v) identified in bone marrow macrophages (4).

Consistent with the effect of *Tshr* deficiency, the administration of recombinant TSH to rodents or people prevents bone loss not only by inhibiting bone resorption, but also by stimulating bone formation (2, 8, 9). The antiresorptive effects are exerted through a low-abundance G protein-coupled *Tshr* on the osteoclast precursor, the activation of which suppresses c-Jun N-terminal kinase (Jnk) to directly inhibit osteoclastogenesis, as well as reduces the release of the osteoclastogenic cytokine TNF $\alpha$  (1). TSH also promotes bone formation by stimulating osteoblast differentiation primarily through the activation of protein kinase C $\delta$  and the up-regulation of the noncanonical Wingless/Integrase-1 (Wnt) components frizzled and Wnt5a (10). A Tsh-induced, fast-forward short loop in bone marrow stimulates the production of Wnt5a to

enhance osteoblastogenesis, and of osteoprotegerin, to further inhibit bone resorption as a third mechanism for TSH-mediated osteoclast control (10).

Here, we focus on the central role of TNF $\alpha$  in mediating the action of Tsh on the skeleton for several reasons. First, high circulating TNF $\alpha$  levels in patients with thyrotoxicosis with suppressed TSH levels have been implicated in the bone loss that accompanies the disease (11). Second, the TNF $\alpha$  elevation in human hyperthyroidism is recapitulated in *Tshr*<sup>-/-</sup> mice, in which CD11b<sup>+</sup> macrophages seem to be the predominant source (12). Third, in preliminary studies, the deletion of *Tnfa* reversed the increased osteoclastogenesis in *Tshr*<sup>-/-</sup> and *Tshr*<sup>+/-</sup> mice in ex vivo cultures (12). Finally, Tsh inhibits cytokine-induced *Tnfa* expression by bone marrow macrophages, as does the overexpression of a constitutively active *Tshr* in transgenic mice (12). Taken together, the data strongly suggest a role for TNF $\alpha$ , as well as other related downstream cytokines, such as IL-1 and IL-6, in Tsh action on bone.

In this study, we provide definitive evidence that the osteoporosis due to *Tshr* deficiency, at least in part, arises from elevated *Tnfa* expression. Notably, the genetic deletion of *Tnfa* fully rescues the reduced bone formation, increased bone resorption, and osteoporosis observed in *Tshr*<sup>-/-</sup> and *Tshr*<sup>+/-</sup> mice. We also explore in vitro whether bone-marrow-derived TNF $\alpha$  and Tsh- $\beta$ v interact in the local control of bone remodeling.

## Results

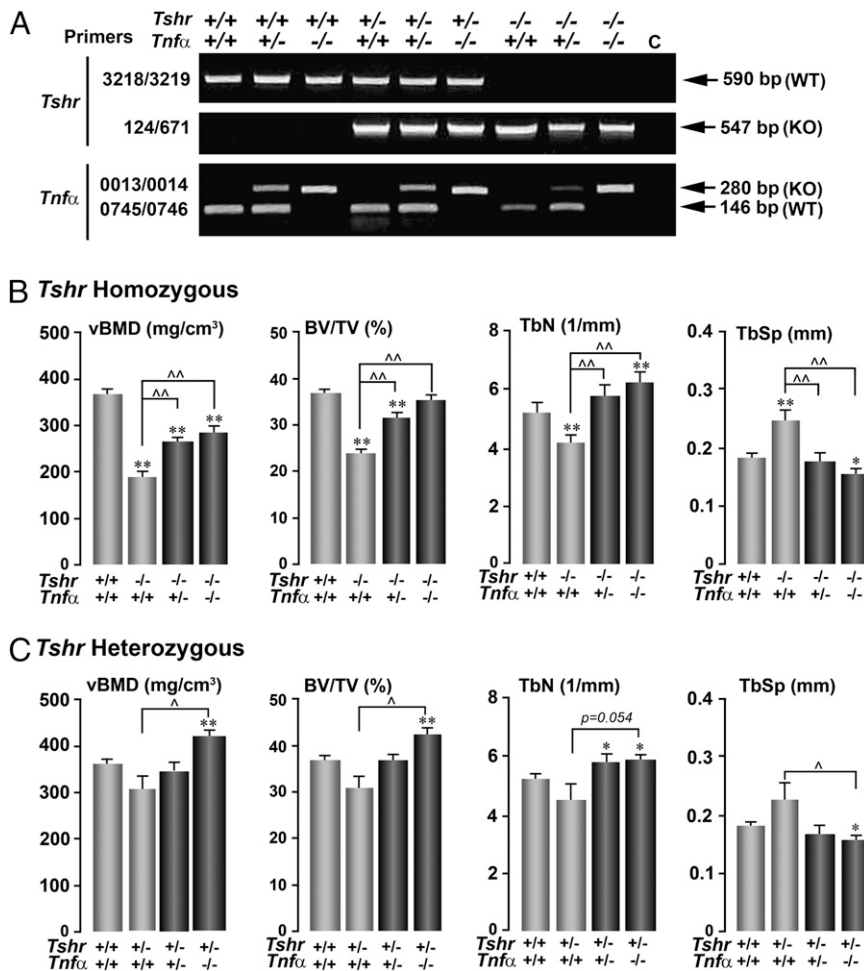
To explore a pathophysiologic role for TNF $\alpha$  in causing the osteoporosis of TSHR deficiency, we crossed *Tshr*<sup>+/-</sup> and *Tnfa*<sup>+/-</sup> mice to yield compound mutants, the genotype of which was confirmed by PCR (Fig. 1A). The focus was to examine for reversal of the bone phenotype of *Tshr* deficiency in the absence of TNF $\alpha$ . Mutants on the *Tshr*<sup>-/-</sup> background were fed on thyroid chow to ensure that they would remain euthyroid and grow and reproduce normally. All mice were on a B6/129 background. At 12 wk of age, the mice were injected with calcein (15 mg/kg) at days -7 and -2 before they were killed to label bone-forming surfaces. Structural and dynamic bone remodeling parameters were measured on trabecular bone by microtomography (L5) and histomorphometry (L1–L3), respectively (13).

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Conflict of interest statement: M.Z. is a named inventor of a pending patent application related to osteoclastic bone resorption filed by the Mount Sinai School of Medicine.

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**Fig. 1.** Deletion of *Tnfα* rescues the osteoporosis owing to *Tshr* deficiency. Agarose gels showing genotype of representative compound mutants, using specific primer sets to detect *Tshr* and *Tnfα* gene expression. (A) The effect of deleting the *Tnfα* gene on a homozygote (*Tshr*<sup>-/-</sup>) (B) or heterozygote (*Tshr*<sup>+/-</sup>) (C) background on microtomography-based parameters of trabecular architecture, namely vBMD, BV/TV, TbN, and TbSp. Statistics: Student *t* test with Bonferroni's correction; comparisons as shown, \**P* ≤ 0.05; \*\**P* ≤ 0.01, compared with *Tshr*<sup>+/-</sup>/*Tnfα*<sup>+/+</sup> mice; ^*P* ≤ 0.05; ^^*P* ≤ 0.01, comparisons with *Tshr*<sup>-/-</sup>/*Tnfα*<sup>+/+</sup> (B) or *Tshr*<sup>+/-</sup>/*Tnfα*<sup>+/+</sup> (C) mice; mean ± SEM is shown, up to eight mice per group.

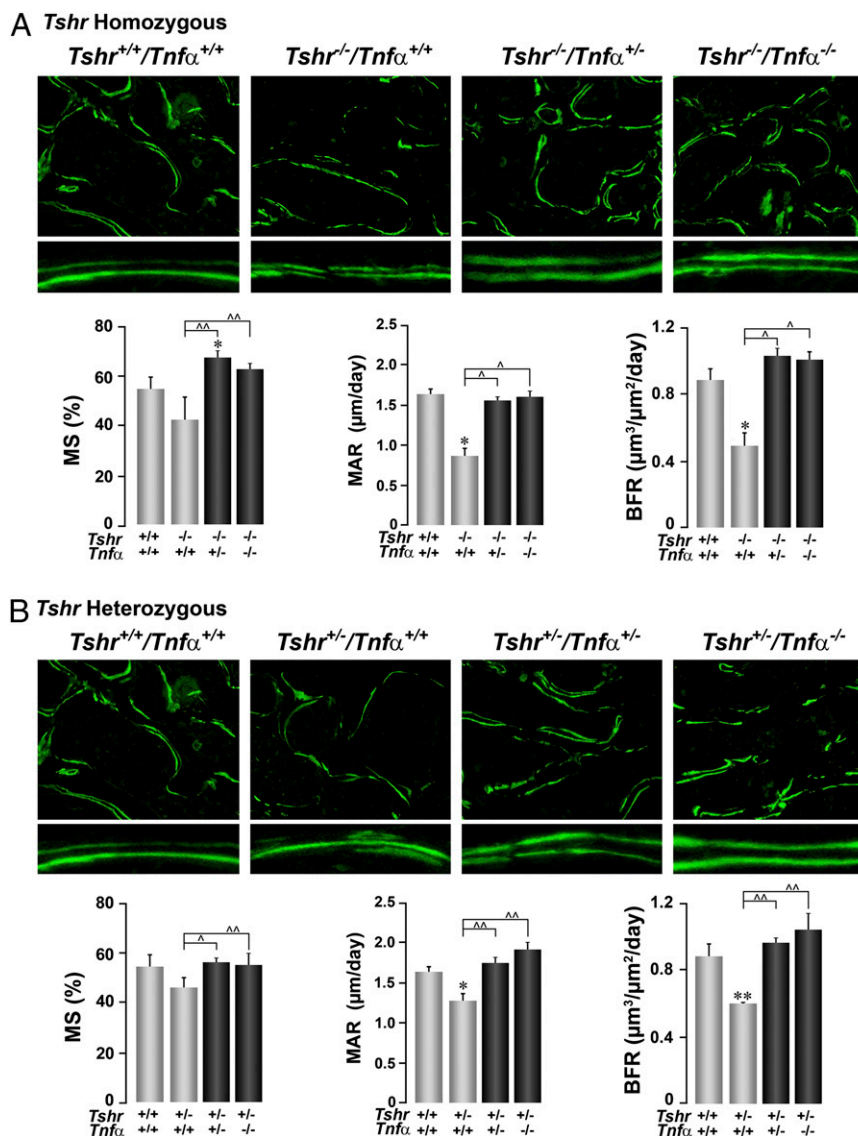
We found that *Tshr*<sup>-/-</sup>/*Tnfα*<sup>+/+</sup> mice showed a significantly lower volumetric bone mineral density (vBMD), bone volume/trabecular volume (BV/TV), and trabecular number (TbN) and a correspondingly higher trabecular spacing (TbSp) compared with wild-type controls (Fig. 1B). However, either a ~50% reduction of *Tnfα* in *Tshr*<sup>-/-</sup>/*Tnfα*<sup>+/-</sup> mice or a complete loss of *Tnfα* in double-homozygote *Tshr*<sup>-/-</sup>/*Tnfα*<sup>-/-</sup> mice rescued this lower vBMD, BV/TV, and TbN and higher TbSp (Fig. 1B). The effect of deleting the *Tshr* on one allele was less marked than that noted in homozygous mice, confirming previous results (1). However, the osteopenic phenotype in the compound heterozygote *Tshr*<sup>+/-</sup>/*Tnfα*<sup>+/-</sup> was rescued fully, with an overshoot in *Tshr*<sup>+/-</sup>/*Tnfα*<sup>-/-</sup> mice (Fig. 1C).

Low bone mass occurs when the process of bone resorption exceeds bone formation (14). In the case of *Tshr* deficiency, there was an uncoupling between bone formation and resorption: Whereas bone formation was lower than in controls, resorption was higher, leading to lower BV/TV (Figs. 2 and 3).

Parameters of bone formation, notably bone formation rate (BFR) and mineral apposition rate (MAR), were significantly lower in both heterozygote (*Tshr*<sup>+/-</sup>/*Tnfα*<sup>+/+</sup>) and homozygote (*Tshr*<sup>-/-</sup>/*Tnfα*<sup>+/+</sup>) mice, compared with wild-type littermates (Fig. 2). Mineralizing surfaces (MSs) showed only a trend (Fig. 2). However, even a 50% deletion of *Tnfα* in either genotype reversed

the decrements in MSs, MAR, and BFR to levels that were not significantly different from those of control littermates, with the exception of an overshoot in MSs in *Tshr*<sup>-/-</sup>/*Tnfα*<sup>+/-</sup> mice and MAR and BFR in *Tshr*<sup>+/-</sup>/*Tnfα*<sup>-/-</sup> mice. That the deletion of *Tnfα* rescued the *Tshr*<sup>-/-</sup> phenotype in a graded, dose-related manner suggested that the low bone formation in *Tshr* deficiency was caused by elevated *Tnfα* levels. This is consistent with the fact that *Tnfα* is known to cause osteoblast apoptosis (15).

In contrast to its proapoptotic action on osteoblasts (15), *Tnfα* increases osteoclast formation (16, 17). We have previously shown that increases in osteoclastogenesis *ex vivo* in bone marrow cultures derived from *Tshr*-deficient mice is reversed in the absence of *Tnfα* (12). Here, we show that whereas tartrate-resistant acid phosphatase (TRAP)-positive resorbed surfaces *in vivo* are significantly elevated in *Tshr*<sup>-/-</sup> mice (*P* = 0.019), there is strong inhibition of resorption in *Tshr*<sup>-/-</sup>/*Tnfα*<sup>+/-</sup> and *Tshr*<sup>-/-</sup>/*Tnfα*<sup>-/-</sup> mice (Fig. 3A). Notable is that the deletion of *Tnfα* not only rescued the hyperresorption phenotype of *Tshr*<sup>-/-</sup> mice, but also suppressed resorption to levels lower than control littermates in both *Tshr* homozygote and heterozygote mice (Fig. 3). Overall, therefore, elevated *Tnfα* levels in *Tshr* deficiency (1) not only seem to inhibit bone formation, but also to elevate resorption, thus causing osteopenia. Deletion of *Tnfα* on a *Tshr*-deficient background reverses the phenotype. These genetic



**Fig. 2.** Deletion of *Tnfa* rescues the low bone formation owing to *Tshr* deficiency. The effect of deleting the *Tnfa* gene on a homozygote (*Tshr*<sup>-/-</sup>) (A) or heterozygote (*Tshr*<sup>+/-</sup>) (B) background on bone formation, shown either as double-labeled surfaces (10 $\times$ , Lower: magnified digitally to show differences in interlabel distances) or as quantitative estimates of MS, MAR, and BFR (units as shown). Statistics by Student *t* test with Bonferroni's correction; comparisons as shown, \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ , compared with *Tshr*<sup>+/+</sup>/*Tnfa*<sup>+/+</sup> mice;  $\Delta P \leq 0.05$ ;  $\Delta\Delta P \leq 0.01$ , comparisons with *Tshr*<sup>-/-</sup>/*Tnfa*<sup>+/+</sup> (A) or *Tshr*<sup>+/-</sup>/*Tnfa*<sup>+/+</sup> (B) mice; mean  $\pm$  SEM is shown, 9–57 sections from up to six mice per group.

rescue studies implicate *Tnfa* in the pathophysiology of bone loss arising from low Tsh signaling.

We carried out *in vitro* assays to examine individual interactions between Tsh and *Tnfa*. We found that recombinant TSH (1 and 10 mU/mL) inhibited osteoclastogenesis in bone marrow cell cultures derived from wild-type mice, but the effect was expectedly abolished in cultures from *Tshr*<sup>hyt/hyt</sup> mice that express a signaling-deficient *Tshr* (Fig. 4A). We next examined whether the action of *Tnfa* on osteoclastogenesis was exerted independently of the *Tshr*. Fig. 4B shows that *Tnfa* (50 ng/mL) stimulated osteoclast formation both in wild-type and *Tshr*<sup>hyt/hyt</sup> mice.

We showed that TSH (0.1–10 mU/mL) inhibited both basal and *Tnfa*-induced *Tnfa* expression (Fig. 4C and D). However, TSH stimulated, rather than inhibited, *Tnfa* expression from embryonic stem (ES) cell-derived osteoblasts (10) (Fig. 4E). These data argue for a counterregulatory mechanism in which Tsh will paradoxically inhibit and stimulate *Tnfa* expression from

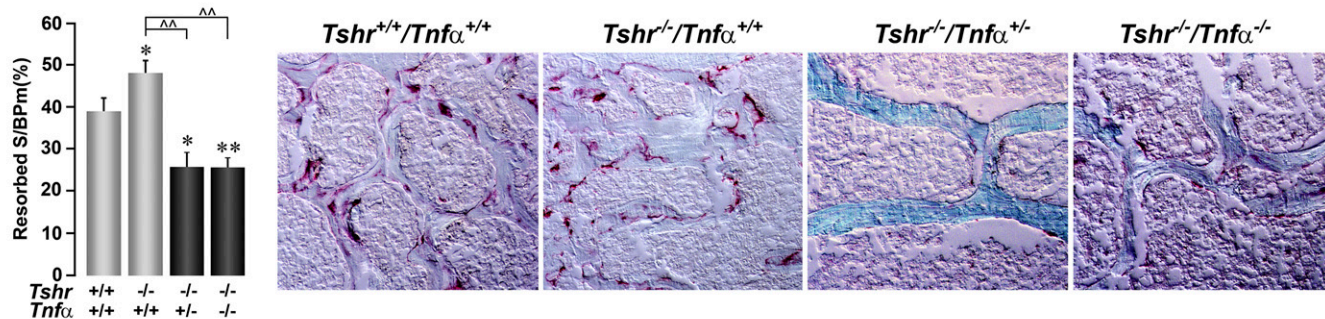
osteoclasts and osteoblasts, respectively. Finally, *Tnfa* markedly stimulated the production of the alternatively spliced product of the *Tsh* gene, Tsh- $\beta$ v, from macrophages (Fig. 4F). We thus surmise that *Tnfa* produced by the osteoblast in response to Tsh could potentially stimulate the production of Tsh- $\beta$ v as a feedback mechanism for osteoprotection.

## Discussion

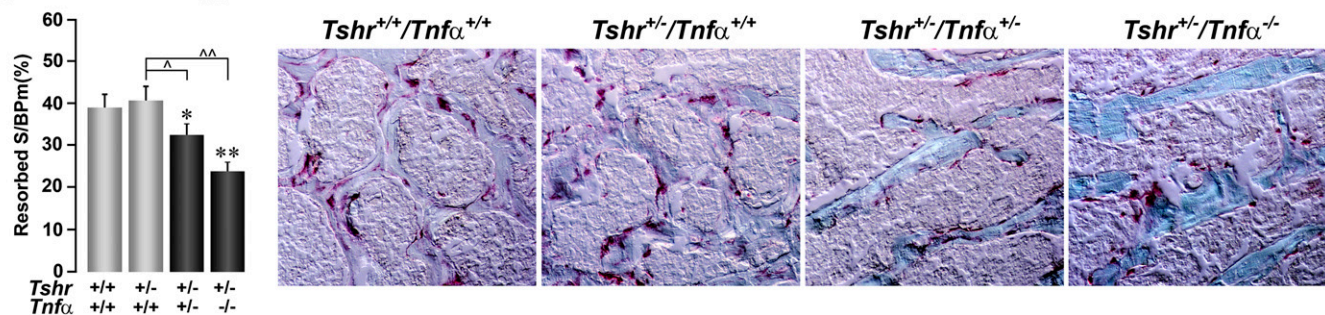
We have shown that *Tshr* deficiency up-regulates and Tsh down-regulates *Tnfa* expression by modulating activator protein 1- and NF- $\kappa$ B-mediated transcriptional activation of the *Tnfa* gene (1, 12). This action is mediated through a receptor activator of NF- $\kappa$ B ligand (RANKL)-responsive *cis*-acting regulatory element (CCG AGA CAG AGG TGT AGG GCC) spanning from -157 to -137 bp of the 5'-flanking region of the *Tnfa* gene (18). The latter sequence binds two high-mobility group box proteins, Hmgb1 and Hmgb2, that are down-regulated by Tsh (18). Here, we show that the genetic ablation of *Tnfa* in *Tshr*-deficient



### A *Tshr* Homozygous



### B *Tshr* Heterozygous



**Fig. 3.** Deletion of *Tnfα* suppresses resorption. The effect of deleting the *Tnfα* gene on a homozygote (*Tshr*<sup>-/-</sup>) (A) or heterozygote (*Tshr*<sup>+/-</sup>) (B) background on bone resorption, shown either as TRAP-positive surfaces (10 $\times$ ; dark pink) or as quantitative estimates of resorption surfaces (resorbed S/BPm). Statistics by Student *t* test with Bonferroni's correction; comparisons as shown, \**P*  $\leq$  0.05; \*\**P*  $\leq$  0.01, compared with *Tshr*<sup>+/+</sup>/*Tnfα*<sup>+/+</sup> mice;  $\Delta$ *P*  $\leq$  0.05;  $\Delta\Delta$ *P*  $\leq$  0.01, compared with *Tshr*<sup>-/-</sup>/*Tnfα*<sup>+/+</sup> (A) or *Tshr*<sup>+/-</sup>/*Tnfα*<sup>+/+</sup> (B) mice; mean  $\pm$  SEM is shown, 10–134 sections from up to 12 mice per group.

mice rescues, in a graded, dose-dependent manner, the osteoporosis arising from increased bone resorption and decreased bone formation. Together, the data establish a fundamental role for *Tnfα* in the bone loss that accompanies *Tshr* deficiency. In contrast, we have shown previously that the related glycoprotein *Fsh* directly stimulates *Tnfα* production, and that mice lacking *Fshβ* have low circulating *Tnfα* levels (17). This would implicate *TNFα* as being central to the opposing actions of the two glycoprotein hormones *Tsh* and *Fsh*.

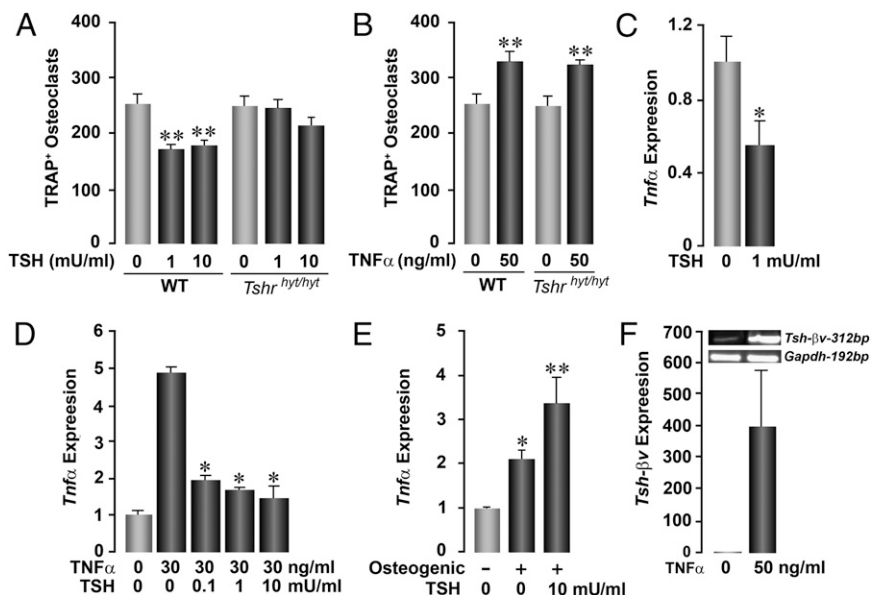
There is no doubt that *Tnfα* increases osteoclastic bone resorption: Most *Tnfα*-driven diseases, such as rheumatoid arthritis and Crohn disease, are characterized by profound local osteolysis, as well as systemic osteoporosis (19). One mechanism is the direct stimulatory action of *Tnfα* on osteoclast precursor differentiation, so that more mature, bone-resorbing cells are produced, as is noted in the *Tnfα* transgenic mouse (16, 20). In addition, we find that *Tnfα* increases the size of the osteoclast precursor pool—otherwise, the pool will ultimately deplete as a function of enhanced differentiation, a premise that is not supported either by mathematical modeling or by examining experimental data in *Tnfα* transgenic mice (17).

High T-cell-derived *Tnfα* levels have also been implicated in the pathogenesis of ovariectomy-induced bone loss in mice and in postmenopausal osteoporosis. Notably, *Tnfα* null mice are resistant to ovariectomy-induced bone loss (21), and elevated *TNFα* production from T cells has been documented in postmenopausal women (22). Furthermore, FSH, which is elevated postovariectomy and in postmenopausal women, stimulates *Tnfα* production (17), suggesting that *Tnfα* may be a common signal that drives, at least part, the bone loss seen postmenopause and in hyperthyroid states. This may be of therapeutic relevance because more than 10% of postmenopausal women receive thyroid hormone replacement therapy, which often causes subclinical hyperthyroidism, essentially characterized by low TSH levels (23).

Furthermore, hyperthyroidism is also 10-fold more common in women (23). The question thus arises whether the two diseases together could produce more profound bone loss, and whether, by implication, anti-TNF therapies may have a role. Alternatively, in view of our observation that *Tsh* is both antiresorptive and anabolic (2), although admittedly ambitious, it is possible that novel thyroid-sparing TSH analogs may become therapeutic options for human osteoporosis.

Mechanistically, we surmise that the elevated *Tnfα* in *Tshr*<sup>-/-</sup> mice will increase osteoclast formation and contribute to bone loss via several interacting mechanisms (Fig. 5). First, it will act directly on precursors to stimulate osteoclastogenesis (16, 17). Second, it will create a footprint for the release of other pro-osteoclastogenic cytokines, such as IL-1 and IL-6, toward a feed-forward loop to enable further *Tnfα* production and osteoclast formation (24). Third, we show that although excessive *Tnfα* can stimulate *Tsh-βv* production from macrophages (Fig. 4F); because of the absence of the *Tshr*, the released molecule will fail to stimulate the production of osteoprotegerin, a Rank-1 inhibitor, from osteoblasts, an action that we have previously documented (10). Finally, released *Tsh-βv* will itself not be able to inhibit osteoclastic bone resorption in *Tshr*<sup>-/-</sup> mice. Hence, profound hyperresorption and bone loss should, and does, follow. When *Tnfα* is removed from the *Tshr*<sup>-/-</sup> background, its effect on osteoclastogenesis is removed, resulting in a greater than full recovery, as noted, of the hyperresorption.

In contrast to its action on the osteoclast, the effect of *Tnfα* on the osteoblast is conflicting. *Tnfα* has been shown to inhibit osteoblast differentiation to a mineralizing phenotype and to stimulate apoptosis in vitro (15, 25). Specifically, *Tnfα* inhibits osteoblastic function by activating NF- $\kappa$ B and repressing transcription of the osteocalcin gene (26, 27). However, these effects cannot explain the high bone formation noted in *Tnfα* transgenic mice (20). In these mice, osteoblast numbers are doubled,



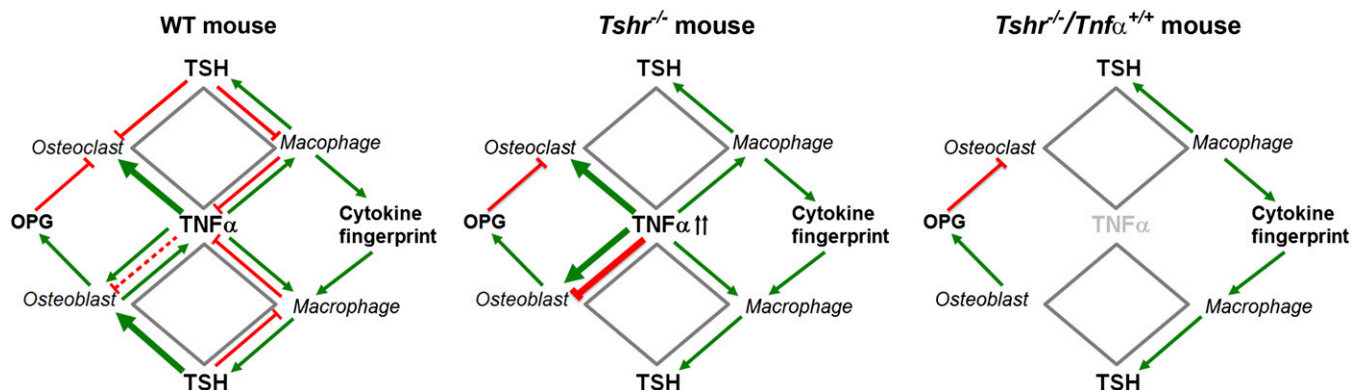
**Fig. 4.** Interactions between Tsh and Tnf $\alpha$ . Effect of Tsh (A) and Tnf $\alpha$  (B) on TRAP-positive osteoclast formation in ex vivo bone marrow cell cultures derived from WT mice or *Tshr<sup>hyt/hyt</sup>* mice, which harbor a mutated, signaling-deficient Tshr. Whereas TSH inhibits osteoclast formation in WT cultures, the effect is lost in cultures from *Tshr<sup>hyt/hyt</sup>* mice (A). In contrast, Tnf $\alpha$  stimulates osteoclastogenesis in both WT and *Tshr<sup>hyt/hyt</sup>* mice (B). TSH inhibits both basal (C) and Tnf $\alpha$ -induced (D) Tnf $\alpha$  expression in osteoclasts, whereas it stimulates Tnf $\alpha$  expression by osteoblasts differentiating in osteogenic media (10) (E) (by quantitative PCR). Tnf $\alpha$  stimulates the expression of Tsh- $\beta$ v in RAW264.7 macrophages, shown as 312-bp product on agarose gel and quantitated by quantitative PCR (F). Statistics by Student t test with Bonferroni's correction; all comparisons with zero dose, \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ .

suggesting that excessive Tnf $\alpha$  can be pro-osteoblastic in vivo. Unexpectedly, we note a reduction, rather than a stimulation, of bone formation in *Tshr<sup>-/-</sup>* mice that overexpress Tnf $\alpha$ . This may result from the absence of the direct osteoblastogenic action of Tsh, or be due to predominance of potential antiosteoblastic effects of Tnf $\alpha$  (15, 25) in the face of Tshr deficiency. Although unlikely, the latter mechanism could explain full rescue of the bone formation phenotype in the *Tshr<sup>-/-</sup>/Tnf $\alpha$ <sup>-/-</sup>* double-mutant mice.

Another important consideration relates to the critical role of Tsh- $\beta$ v produced in bone marrow in skeletal regulation. Our observation that hyperthyroid *Tshr<sup>-/-</sup>* mice lost more bone than hyperthyroid mice with suppressed circulating Tsh levels argues strongly for osteoprotection by a Tsh-like molecule locally within bone marrow. This makes biological sense because, unlike

pituitary Tsh, Tsh- $\beta$ v in macrophages is regulated positively, rather than negatively, by thyroid hormones. Thus, we find that excess thyroid hormone stimulates Tsh- $\beta$ v expression, which then protects the skeleton by acting on bone-cell Tshrs (Fig. 5). The effects of this osteoprotection are lost in *Tshr<sup>-/-</sup>* mice, culminating in significant bone loss.

Finally, the expression of so-called pituitary hormones and their receptors in bone, and at other hitherto unrecognized sites, has only been recognized recently. Osteoblast-like cells were shown to possess Tshrs, as were T cells and enterocytes, mainly through early radiolabeling studies (28, 29). However, the significance of Tshrs in nonthyroid tissues remained unclear until 2003, when we showed that functional Tshrs were indeed expressed on bone cells (1). Thereafter, we and others showed that functionally active Fshrs (30), melanocortin 2 receptors (31, 32),



**Fig. 5.** A central role for Tnf $\alpha$  in hyperthyroid bone loss. Elevated Tnf $\alpha$  in *Tshr<sup>-/-</sup>* mice increases osteoclastogenesis by acting directly on the osteoclast precursor and by creating a footprint for the release of other pro-osteoclastogenic cytokines, such as IL-1 and IL-6. Excessive Tnf $\alpha$  also stimulates Tsh- $\beta$ v production from macrophages, but in *Tshr<sup>-/-</sup>* mice the released molecule fails to inhibit osteoclastic bone resorption or to stimulate the production of osteoprotegerin, a Rank-1 inhibitor. The Tsh- $\beta$ v also fails to stimulate osteoblastic bone formation in *Tshr<sup>-/-</sup>* mice. Hence, profound hyperresorption, reduced bone formation, and bone loss ensue in Tsh, signaling deficiency. When Tnf $\alpha$  is removed from the *Tshr<sup>-/-</sup>* background, full recovery occurs.



and oxytocin (33, 34) receptors are expressed on bone cells. Notably, the bone cell *Fshr* is a shorter isoform missing exon 9 (35). Likewise, and importantly, we also found that the ligands of certain of these receptors, such as oxytocin and Tsh- $\beta$ v, are expressed in bone cells (34, 36). We believe, therefore, that it is critical to begin to study local regulatory control mechanisms through which "pituitary" glycoprotein hormones interact with cytokines, such as Tnf $\alpha$  and others, in the local, as opposed to the central, control of bone remodeling and bone mass. Important therapeutic implications may eventually follow.

## Materials and Methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine. We genotyped groups of compound mutant mice on a B6/129 background in which the *Tshr* and/or *Tnf $\alpha$*  genes were deleted (37) (Jackson Laboratory) (Fig. S1). For microtomography measurements, the L5 vertebra was scanned non-destructively, as before (13), by using a Scanco microtomography scanner ( $\mu$ CT-40; Scanco Medical) at 12- $\mu$ m isotropic voxel size, with an X-ray source power of 55 kV and 145  $\mu$ A, and integration time of 300 ms. The trabecular microstructure of the entire secondary spongiosa of L5 between the cranial and the caudal area was evaluated. The scanned grayscale images were processed by using a low-pass Gaussian filter to remove noise, and a fixed bone mineral density (BMD) threshold of 220 mg/cm<sup>3</sup> was used to extract the mineralized bone from soft tissue and the marrow phase. The same

settings for scan and analysis were used for all samples. Trabecular bone parameters included volumetric BMD, BV/TV, TbN, and TbSp (units in Fig. 1).

Bone formation and resorption rates were quantified by dynamic histomorphometry following two sequential injections of calcein (15 mg/kg) 5 d apart before animals were killed (at days -7 and -2). Measured parameters included MS, MAR, BFR, and TRAP surfaces (resorbed S/BPm) (as in ref. 31). Histological sections were photographed on a fluorescent microscope (Observer Z1; Zeiss) (34).

For the ex vivo studies, bone marrow was isolated and stromal cell cultures performed in the presence of ascorbate-2-phosphate (1 mM) for 10 d (30). Additionally, osteoclast formation assays were performed following Ficoll purification of bone marrow and incubation with macrophage colony-stimulating factor (M-CSF) (30 ng/mL) and RANK-L (50 ng/mL) for 5 d (30). For RAW264.7 macrophages, we excluded M-CSF from the 5-d cultures. Quantitative PCR was performed on extracted RNA using appropriate primer sets, as described previously (34). Mouse ES cell cultures were performed as described previously (10, 38).

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