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Evidence that the rat osteopetrotic mutation toothless (tl) is not in the TNFSF11 (TRANCE, RANKL, ODF, OPGL) gene

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Evidence that the rat osteopetrotic mutation *toothless (tl)* is not in the *TNFSF11 (TRANCE, RANKL, ODF, OPGL)* gene

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ABSTRACT The *toothless (tl)* osteopetrotic mutation in the rat affects an osteoblast-derived factor that is required for normal osteoclast differentiation. Although the genetic locus remains unknown, the phenotypic impact of the *tl* mutation on multiple systems has been well characterized. Some of its actions are similar to *tumor necrosis factor superfamily member 11 (TNFSF11)*; also called *TRANCE, RANKL, ODF* and *OPGL* null mice. *TNFSF11* is a recently described member of the tumor necrosis factor superfamily which, when expressed by activated T cells, enhances the survival of antigen-presenting dendritic cells, and when expressed by osteoblasts, promotes the differentiation and activation of osteoclasts. The skeletal similarities between *tl* rats and *TNFSF11(-/-)* mice include 1) profound osteoclastopenia (*TNFSF11*-null mice, 0% and *tl* rats 0-1% of normal); 2) persistent, non-resolving osteopetrosis that results from 3) a defect not in the osteoclast lineage itself, but in an osteoblast-derived, osteoclastogenic signal; and 4) a severe chondrodysplasia of the growth plates of long bones not seen in other osteopetrotic mutations. The latter includes thickening of the growth plate with age, disorganization of chondrocyte columns, and disturbances of chondrocyte maturation. These striking similarities prompted us to undertake studies to rule in or out a *TNFSF11* mutation in the *tl* rat. We looked for expression of *TNFSF11* mRNA in *tl* long bones and found it to be over-expressed and of the correct size. We also tested *TNFSF11* protein function in the *tl* rat. This was shown to be normal by flow cytometry experiments in which activated, spleen-derived T-cells from *tl* rats exhibited normal receptor binding competence, as measured by a recombinant receptor assay. We also found that *tl* rats develop histologically normal mesenteric and peripheral lymph nodes, which are absent from *TNFSF11*-null mice. Next, we found that injections of recombinant *TNFSF11*, which restores bone resorption in null mice, had no therapeutic effect in *tl* rats. Finally, gene mapping studies using co-segregation of polymorphic markers excluded the chromosomal region containing the *TNFSF11* gene as harboring the mutation responsible for the *tl* phenotype. We conclude that, despite substantial phenotypic similarities to *TNFSF11(-/-)* mice, the *tl* rat mutation is not in the *TNFSF11* locus, and that its identification must await the results of further studies.

KEY WORDS: *bone resorption, osteopetrosis, osteoclast, gene mapping, chondrodysplasia*

Introduction

Osteopetrosis, or marble bone disease, is an inherited, pathological increase in bone mass that results from impaired bone resorption by osteoclasts. Depending upon the mutated gene, osteopetrotic phenotypes vary widely in severity, in age of onset (or, in some cases, of spontaneous recovery), and in the extent to which tissues and organs other than bone are affected (Popoff and Marks, 1995). While most known mutations impact genes expressed by the osteoclasts or their progenitors, two known factors

expressed by osteoblasts are required to induce normal osteoclast formation and activation, colony stimulating factor-1 (CSF-1 or M-CSF; Yoshida *et al.*, 1990) and the tumor necrosis factor superfamily

Abbreviations used in this paper: CSF-1, colony stimulating factor 1, also known as m-CSF; RT-PCR, reverse transcription polymerase chain reaction; nlm, normal littermate; *tl*, *toothless* osteopetrotic mutation; TNFRSF11A, tumor necrosis factor receptor superfamily member 11A, also known as TRANCE receptor and RANK; TNFSF11, tumor necrosis factor superfamily member 11 –also known as TRANCE, RANKL, ODF and OPGL; TRAP, tartrate-resistant acid phosphatase.

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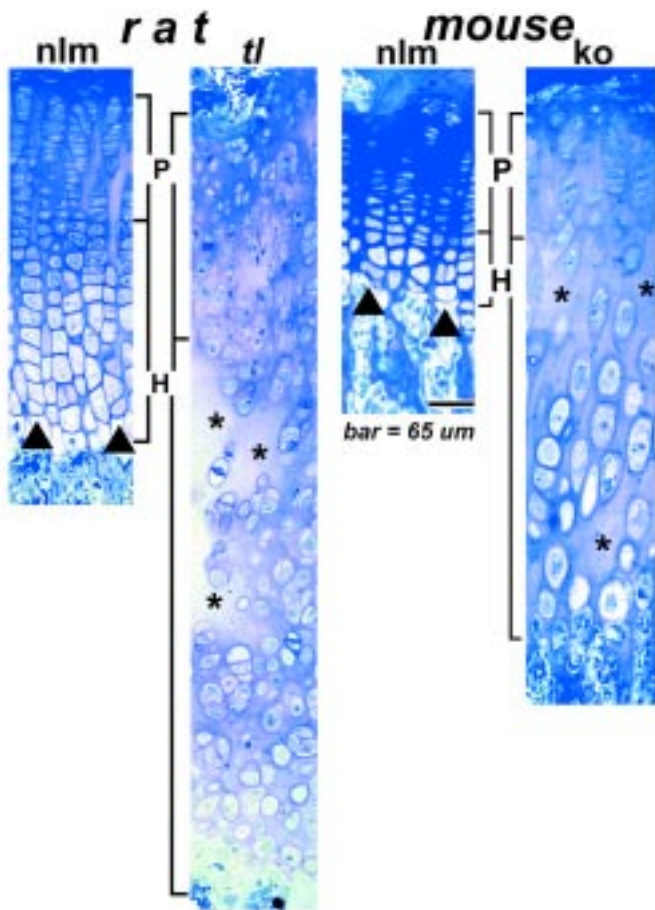


Fig. 1. Similar chondrodysplasias in *tl* rat and *TNFSF11*^{-/-} mice. One of several phenotypic similarities is seen in proximal tibial growth plates of 4-week-old animals, with abnormal thickening of growth plates, disorganized chondrocyte columns, and failure to form normal proliferating (P) or hypertrophic (H) zones. (Left panels) Rat normal littermate (nlm) and *tl* mutant (*tl*). (Right panels) Mouse normal littermate (nlm) and *TNFSF11* knockout (*ko*). Both mutations also show areas of hypocellularity (asterisks), and lack thin transverse septae at the chondroosseous junction (arrowheads in normals). Of the known osteopetrotic mutations, only genes in the *TNFSF11* osteoclast differentiation pathway have been shown to produce this growth plate phenotype. (2 μ m Epon sections, toluidine blue stained).

ily member *TNFSF11* [(Kim *et al.*, 2000; Kong *et al.*, 1999); see Locksley *et al.*, 2001 for the most recent standardized nomenclature for the TNF and TNF receptor superfamilies] which has been named, in chronological order, TRANCE (Wong *et al.*, 1997), RANKL (Anderson *et al.*, 1997), ODF (Yasuda *et al.*, 1998) and OPGL (Kong *et al.*, 1999).

The *toothless* (*tl*) mutation in the rat produces severe osteopetrosis that is inherited in an autosomal, recessive manner (Cotton and Gaines, 1974). Unlike many other osteopetrotic mutations, the *tl* rat is not cured by hematopoietic stem cell transplantation from its normal littermates (Marks, 1977), nor are *tl*-derived osteoblasts capable of activating co-cultured normal osteoclasts (Hermey *et al.*, 1996; Sundquist *et al.*, 1995). This means that the defective gene is not a product of the osteoclast/monocyte/macrophage lineage, but rather is an osteoblast-expressed factor. *tl*

rats have profound osteoclastopenia which contrasts, for example, with the *incisor absent* (*ia*) mutation in which osteoclasts are present at 2-3 times the normal numbers, but are small and non-functional (Odgren and Marks, 1998). *tl* rats are also affected by severe chondrodystrophy of the growth plate, with thickening of the central area over time, disorganized chondrocyte columns, and failure of growth cartilage to mineralize (Odgren *et al.*, 1999; Seifert, 1996).

We noted phenotypic manifestations of the *tl* mutation that bore striking similarities to *TNFSF11*-null mice. First, *TNFSF11* is produced in the skeleton by osteoblasts. Second, *TNFSF11*-null mice have severe osteopetrosis that does not resolve with age. Third, null mice have a profound deficiency of osteoclasts, with none found in osteoclast-specific histologic staining of bone sections (Kim *et al.*, 2000; Kong *et al.*, 1999). Finally, the growth plates of null mice are dystrophic, a feature not seen in most other osteopetrotic mutations. Chondrocytes are disorganized, with central thickening of the growth plates and highly abnormal differentiation progression (Kim *et al.*, 2000), much as is seen in the *tl* rat (Odgren *et al.*, 1999; Seifert, 1996). These observations led us to undertake investigations to test the hypothesis that the *tl* rat is a loss-of-function mutation in the *TNFSF11* gene.

Results

Figure 1 shows comparative histology of dystrophic growth plates of the *tl* rat and *TNFSF11*-null mice at 4 weeks of age. These images of the central region of the proximal tibial growth plates reveal phenotypic characteristics that motivated the present study. Note the marked increase in thickness of both mutants compared to their normal counterparts. Both mutations show failure to form columns, mis-orientation of groups of cells, and failure to exhibit the normal chondrocyte differentiation zones. The proliferating zone is greatly increased in thickness in both mutants, with persistence of small cells and isogenous groups typical of proliferating chondrocytes well beyond the normal depth. Hypertrophic cells do not reach their fully differentiated appearance in either of these mutations, although near-complete hypertrophy is seen more frequently in the *TNFSF11*-null mouse. Both mutations show areas of growth cartilage that are devoid of cells, suggesting either chondrocyte death or hypersecretion of cartilage matrix, or both. The chondro-osseous junctions on the metaphyseal side of the growth plates are also highly abnormal, and lack the thin, trans-

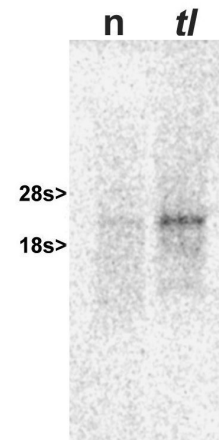


Fig. 2. Northern blot for *TNFSF11* mRNA. 20 μ g of total RNA from long bones of 2-week-old normal (n) and *tl* rats (*tl*) was loaded per lane, and the blot probed with rat *TNFSF11* cDNA. At this loading, normal bone *TNFSF11* mRNA levels is at the detection threshold, while the *tl* bone expressed higher levels of message. Quantitation of signal showed roughly 8-fold up-regulation in the mutant bone. Comparison to rRNA gave an mRNA size of 2.1 kb, in good agreement with the 2.2 kb full-length mouse sequence.

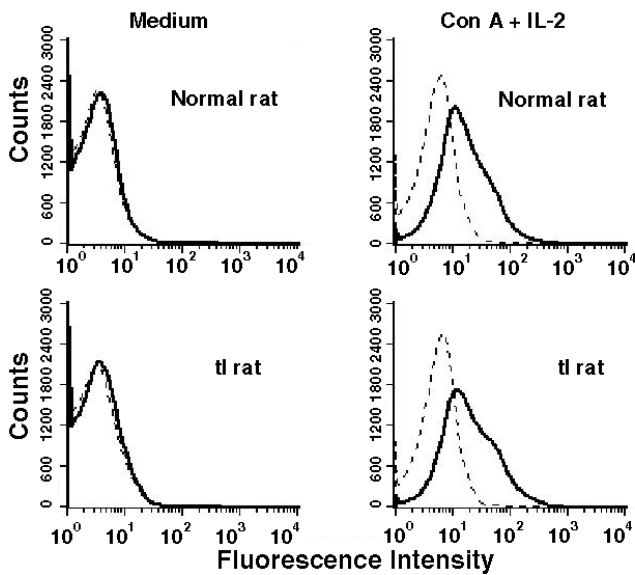


Fig. 3. Flow cytometry reveals functional TNFSF11 expressed by *tl* splenocytes. Spleen cells were stimulated with Concanavalin A and IL-2 for 4 days, probed with hlgG (control, dotted line) or a chimeric protein consisting of TNFSF11 receptor (TRANCE receptor/RANK) fused to human IgG Fc chain (solid line), incubated with PE-conjugated goat anti-hlgG $F(ab)_2$ and binding was analyzed by FACS. No significant difference was seen between the receptors of *tl* rats and those of their normal littermates. Three separate experiments gave similar results.

verse septae between columns of hypertrophic cells that form the locus of normal vascular invasion and conversion to bone. An ultrastructural analysis of these two phenotypes is in progress.

We examined expression in long bones of TNFSF11 mRNA by Northern blot, and the results are shown in Fig. 2. An 808 base-pair rat TNFSF11 cDNA (Gen Bank Accession # AF425669) was obtained by degenerate oligo-nucleotide RT-PCR of normal rat bone RNA that corresponds to bases 932-1740 of the mouse cDNA (accession # AF013170), comprising the distal end of the coding region and the proximal 3'UTR. There is 91% similarity between rat and mouse in this region, with a 42 nucleotide gap from bases 1361-1403 of the mouse sequence. This cDNA was used to probe total RNA from long bones of *tl* rats and their normal littermates, and a single band of roughly 2.1 kb was detected, in good agreement with the 2.2 kb full-length mouse TNFSF11 cDNA (Wong *et al.*, 1997). Interestingly, the normal rat mRNA was barely detectable at the loading used (20 μ g of total long bone RNA per lane), while the *tl* long bones had about 8-fold higher levels when quantitated by phospho-imaging. This does not rule out a minor mutation in TNFSF11 in the *tl* rat, but it does demonstrate that there is neither a large rearrangement nor an inability to express the TNFSF11 mRNA.

To test whether there might be a functional defect in the TNFSF11 protein in the *tl* rat, we performed flow cytometric analysis of spleen-derived activated T-cells. The cells were first exposed to concanavalin A and IL-2 for 4 days to stimulate TNFSF11 expression and then probed with recombinant mouse TNFSF11 receptor fused to human IgG heavy chain, and labeled with fluorescent anti-human IgG (Josien *et al.*, 1999). As seen in Fig. 3A, *tl* splenocytes bind the receptor construct in a manner

indistinguishable from their normal littermates. This result implies that the TNFSF11 protein in *tl* rats is fully able to bind its receptor, and therefore should participate normally in TNFSF11-mediated signaling.

Further confirmation of normal TNFSF11 activity in the *tl* rat is found in the lymphatic system, and is shown in Fig. 4. TNFSF11-null mice have no peripheral or mesenteric lymph nodes (Kim *et al.*, 2000; Kong *et al.*, 1999), whereas dissection of *tl* rats revealed well-developed mesenteric lymph nodes as well as peripheral nodes. Histological sections of mesenteric and peripheral (inguinal, submandibular, and paratracheal) lymph nodes from the *tl* rat are normal, with dense populations of lymphocytes and normal-ap-

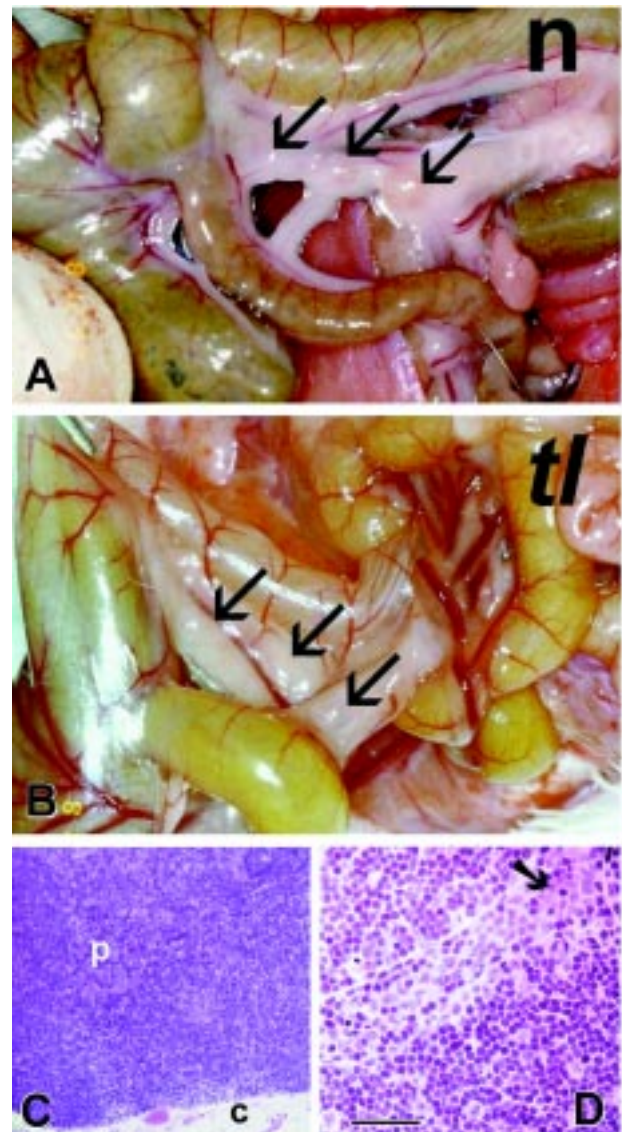


Fig. 4. Normal lymph nodes in *tl* rats. Dissections of the abdominal cavity (A,B) reveal well-developed mesenteric lymph nodes (arrows) in (A) normal (n) and (B) mutant (tl) rats. Histological sections show that inguinal lymph nodes from *tl* rats at (C) low and (D) higher magnification are well-developed, with numerous lymphocytes and normal histological appearance. p, paracortex; c, capsule. Arrow in D indicates sinus in dense region of lymphocytes (3 μ m glycol methacrylate section stained with H&E; bar, 150 μ m in right panel; 600 μ m in left panel).

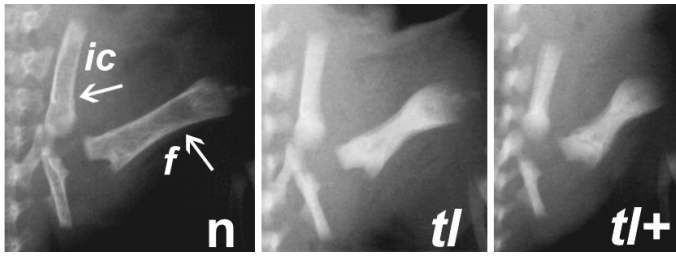


Fig. 5. Treatment of *t/l* rats with recombinant TNFSF11 shows no radiologic improvement. X-rays of normal (*n*), *t/l*, and TNFSF11-treated *t/l* (*t/l+*) rats (1 mg/kg dose) at 10 days post-partum. Normal femur (*f*) and iliac crest (*ic*) are seen in the left panel, with radiolucent marrow spaces, while in both the untreated and treated mutants, there is severe osteopetrosis and no radiological evidence of bone resorption.

pearing stroma and lymphatic ducts. Figure 4 shows cross-sections of inguinal nodes. Similar results were seen in mesenteric and other peripheral nodes (data not shown).

Next, we investigated the effect of treating *t/l* rats with recombinant TNFSF11. Recombinant TNFSF11 (human OPGL, kindly provided by C. Dunstan, Amgen, Inc., Thousand Oaks, CA) was injected subcutaneously into *t/l* rats at 0.1 or 1 mg/kg of body weight every other day for the first 10 post-natal days. X-rays of the treated animals revealed no improvement in their osteopetrotic skeletons (Fig. 5), nor was there any increase in osteoclasts seen in TRAP-stained histological sections of long bones from the treated animals (Fig. 6). Doses of 1 mg/kg rapidly induced osteoclast formation and bone resorption in TNFSF11-null mice, with visible improvement in X-rays (data not shown) and large numbers of osteoclasts in long bones (Fig. 6). Treatment of normal littermates of *t/l* rats with 2 mg/kg every other day for a week caused an increase in metaphyseal osteoclasts and fragility of bones, thus confirming the biological activity of recombinant human TNFSF11 in the rat (not shown).

As the evidence against a TNFSF11 mutation accumulated in the studies described above, we undertook genetic studies to confirm this interpretation. The TNFSF11 gene position has not been directly determined in the rat; nevertheless, it has been mapped in both mouse and human, so its location can be inferred from homology between chromosome regions. In both human and mouse, the TNFSF11 gene is close to the ELF1 gene, on chromosomes 13 and 14, respectively, while in the rat it resides on chromosome 15q. From this region, microsatellite markers were selected for PCR-based segregation analysis as shown in Fig. 7. We outcrossed the *t/l* strain with brown Norway rats, yielding

heterozygous F1 rats. These F1's in turn yielded 11 F2 rats having the *t/l* phenotype whose DNA was analyzed for co-segregation of chromosome 15 markers with the mutation. The results in Fig. 7 show that the TNFSF11-containing region of chromosome 15 and the *t/l* mutation are not linked.

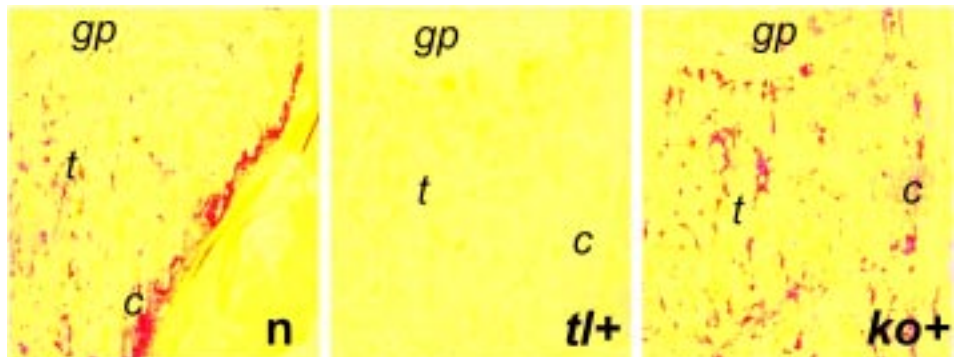
Discussion

The balance between bone formation and bone resorption that is required for normal skeletal and mineral homeostasis, referred to by bone biologists as "coupling," requires both systemic and local factors. The discovery of two osteoblast-derived signaling molecules, CSF-1 and TNFSF11, that are required for normal osteoclast ontogeny and activation represents a great advance in understanding the molecular mechanisms underlying coupling. Interestingly, the phenotype of a naturally-occurring CSF-1-null mutation, the *osteopetrosis* (*op*) mouse (Yoshida *et al.*, 1990), is quite distinct from TNFSF11^(-/-) mutants. Where TNFSF11-null mice have no osteoclasts (Kim *et al.*, 2000; Kong *et al.*, 1999), the *op* mouse does have them, although their numbers are low. The *op* mouse also recovers spontaneously over the first few postnatal months while the TNFSF11-null mouse does not, and there are no growth plate abnormalities in the *op* mouse of the sort shown in Fig. 1. The spontaneous recovery of the *op* mouse suggests the existence of an overlapping or redundant cytokine pathway that can, with time, compensate for the missing CSF-1 in promoting osteoclast differentiation in mice. This is not the case with TNFSF11-null mice nor with *t/l* rats, neither of which show improvement over time.

The discovery of TNFSF11 and the parallels between the *t/l* rat skeletal phenotype and that of TNFSF11-null mice led us to hypothesize that the *t/l* rat may be a naturally-occurring TNFSF11 mutation in the rat. From the data presented in this report, we conclude that this is not the case. First, the *t/l* rat expresses TNFSF11 mRNA in its long bones, and the message is the correct size (Fig. 2). Second, *t/l* rats develop histologically normal mesenteric and peripheral lymph nodes (Fig. 4). Third, the TNFSF11 protein expressed by mitogen-activated *t/l* splenic T-cells is capable of binding to receptor (Fig. 3). Fourth, the *t/l* rat is refractory to injections of recombinant human TNFSF11 sufficient to induce fully functional osteoclasts in knockout mice (Figs. 5 and 6). Finally, segregation analysis of microsatellite markers flanking the TNFSF11 gene enabled us to exclude the primary involvement of this gene (Fig. 7).

Although one expects similar phenotypes in knockout mice for genes that lie downstream in the TNFSF11 signal transduction

Fig. 6. TNFSF11 does not induce osteoclast formation in *t/l* rats. TRAP staining (red) shows abundant osteoclasts in 10-day-old normal (*n*) rat tibia, especially among the metaphyseal trabeculae (*t*) and the cortical region (*c*). No TRAP-positive cells are seen in *t/l* rats treated for 10 days with 1 mg/kg of recombinant TNFSF11 (*t/l+*), while the same regimen induced osteoclast formation and activation in TNFSF11^(-/-) mice (*ko+*). 3 μ m glycol methacrylate tibia sections. *gp* indicates growth plate, for orientation. Original magnification 20 X.



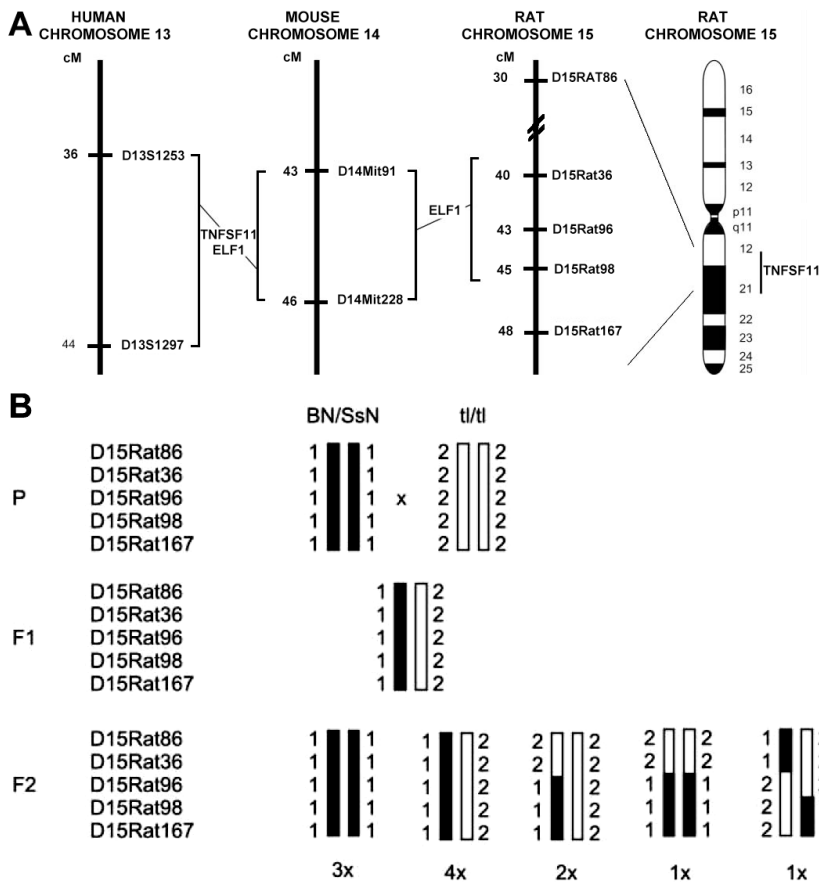


Fig. 7. (A) Chromosomal localization of the rat *TNFSF11* gene and (B) exclusion of the involvement of the *TNFSF11* gene by segregation analysis. (A) No precise localization of the rat *TNFSF11* gene is currently available, but it can be deduced from comparative mapping. Both in human and mouse, the *TNFSF11* gene has been localized close to the *ELF1* gene, respectively on chromosomes 13 and 14. These chromosomal regions are syntenic with rat chromosome 15, which contains the rat *ELF1* gene, specifically within the proximal part of 15q. A set of microsatellite markers spread over this region was selected for analysis. **(B)** The inbred *tl* rat strain was crossed to the inbred BnSsN strain. The black bars indicate the BnSsN haplotype and the white indicate the *tl* haplotype. For informative microsatellite markers selected from the region of chromosome 15, heterozygosity is obtained in the F1 generation. Eleven *tl/tl* animals resulting from intercrosses between F1 animals were analyzed (F2, homozygous for the mutation), and the patterns of inheritance and the number of animals are indicated (3x, etc.). None of the selected markers were found to cosegregate with the *tl* phenotype in all the F2 mutants, indicating that this region does not harbor the disease-containing mutation.

pathway, for example its receptor (TNFRSF11A/TRANCE receptor/RANK) (Li *et al.*, 2000), or its intracellular, receptor-associated factor TRAF6 (Lomaga *et al.*, 1999), these molecules are produced by and act within the osteoclast precursors themselves. The *tl* mutation affects an osteoblast-expressed factor, i.e., *tl* rats are not cured by transplants of normal osteoclast precursor cells (Marks, 1977), nor can *tl* osteoblasts support the activation of normal osteoclasts in co-culture systems (Hervey *et al.*, 1996; Sundquist *et al.*, 1995). Together, these findings demonstrate that the *tl* mutation is not a defect on the osteoclast side of normal osteoblast-osteoclast communication.

The question then arises, what gene is responsible for the *tl* rat phenotype? In addition to *TNFSF11*, the only other osteoblast-derived, osteoclastogenic factor currently known is CSF-1. While CSF-1 injections do restore some osteoclast populations in the *tl* rat, there is no improvement in either the growth plates or long bone growth when *tl* rats are treated with CSF-1 from birth through six weeks (Odgren *et al.*, 1999), nor is there any spontaneous phenotypic improvement seen in *tl* rats over time as there is in the *op* mouse. The gradual recovery of bone resorption in the *op* mouse, in which a frame-shift mutation in the twelfth codon abrogates CSF-1 function (Yoshida *et al.*, 1990), suggests some overlapping, compensatory pathway in cytokine-mediated osteoclast differentiation. These observations, combined with the present data that eliminate *TNFSF11* as the site of the *tl* mutation, raise two possibilities. On the one hand, if the *tl* mutation actually is in the CSF-1 gene, then one must be prepared to account for the widely different phenotypic consequences in the *op* mouse versus the *tl*

rat. On the other hand, there may be a third, osteoblast-derived, osteoclastogenic factor that is mutated in the *tl* rat. Molecular genetic mapping experiments currently underway will help resolve this important question.

Materials and Methods

All animal procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Mutant (*tl/tl*) rats and their normal (+/?) littermates were obtained from inbred colonies maintained for over twenty years at the University of Massachusetts Medical School. Outbreeding of animals of *tl* stock was done by crossing mutant males (genotype *tl/tl*; the trait is inherited in a Mendelian recessive manner) with females of inbred Brown Norway stock (BnSsN; Harlan Sprague Dawley, Indianapolis, Indiana) to obtain F1 heterozygotes. Intercrosses of F1 yielded F2 animals for segregation analyses. The generation of *TNFSF11*^(-/-) mice was previously described (Kim *et al.*, 2000). Colonies of both the rat and mouse strains were maintained under specific-pathogen-free conditions. Mutants were distinguished from normals by neonatal radiography as described (Schneider *et al.*, 1979). In some experiments, *tl* rats, normal littermates, and *TNFSF11*-null mice were treated with recombinant *TNFSF11* (human OPGL, kindly provided by Dr. Colin Dunstan, Amgen, Inc., Thousand Oaks CA). Based on the observations that doses of 0.1 mg/kg, 1 mg/kg, and 2 mg/kg provide, respectively, minimal, profound, and fatal hypercalcemia by 3-4 days in normal mice due to increased bone resorption (Dr. Colin Dunstan, personal communication), we used the following doses in the present study. Mutant rats and null mice were treated with 0.1 or 1 mg/kg body weight, and normal rats received 2

mg/kg, diluted in PBS. The higher dose for normal rats was to verify biologic activity of the recombinant human protein in the rat. Treatment was by subcutaneous injection every other day for 10 days. Control animals received just PBS.

Histology

Dissected bones were stripped of extraneous tissue, fixed overnight in cold 2.5% glutaraldehyde (Polysciences, Warrington PA) and 0.7% ruthenium hexaamine trichloride (Polysciences) in 0.1 M sodium cacodylate and post-fixed for 1-2 hours in 1% osmium tetroxide (Polysciences) in cacodylate (Egglı et al., 1985; Egglı et al., 1988), demineralized in EDTA (10% Na₂EDTA in Tris-HCl, pH 8.0), and embedded in Epon (Polysciences). 2 µm sections were stained with 1% toluidine blue in 1% sodium borate. For histological examination of lymph nodes and for tartrate-resistant acid phosphatase (TRAP) histochemistry staining of osteoclasts, fixation was done overnight in cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate. Bones were demineralized in EDTA and embedded in glycol methacrylate, and 3 µm sections were stained by enzyme histochemistry for TRAP as described (Lindunger et al., 1990). Glycol methacrylate-embedded lymph node sections were stained with hematoxylin and eosin.

RNA analysis

Northern blots were done according to standard methods (Odgren et al., 1996). RNA was isolated from frozen tibiae and femora by pulverization followed by homogenization in Trizol (Life Technologies, Bethesda, MD), and purified as recommended by the manufacturer. 20 µg of total RNA from 4-week-old *tl* rats and their normal littermates was loaded per lane, probed with ³²P-labeled probe (HighPrime kit, Roche, Indianapolis, IN), and scanned with a Molecular Dynamics Storm phospho-imager. The probe was an 808 base-pair cDNA for rat TNFSF11 obtained by RT-PCR from rat bone RNA (GenBank Accession # AF425669). The sequence aligns with bases 932-1740 of the mouse TNFSF11 sequence (GenBank accession # AF013170), with 91% identity and one 42 bp gap from base 1361-1403 in the mouse sequence.

FACS

Flow cytometry was carried out as described (Josien et al., 1999). Briefly, total cells were harvested from spleens of 5-week-old *tl* rats and normal littermates, and 5 X 10⁶ cells were plated on 24-well culture plates in the absence or presence of concanavalin A (5 µg/ml) and IL-2 (10 unit/ml) for 4 days. The expression of TRANCE on activated T cells was examined using either baculovirus-expressed, affinity purified fusion protein of mouse TRANCE receptor (TNFSFR11; RANK) fused to human IgG1 heavy chain or hlgG1 (control) at 5 µg/ml, followed by phycoerythrin (PE)-conjugated goat anti-hlgG (Fc-specific) F(ab')₂ fragment (Caltag, Burlingame, CA).

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