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The osteopetrotic mutation *toothless* (*tl*) is a loss-of-function frameshift mutation in the rat *Csf1* gene: Evidence of a crucial role for CSF-1 in osteoclastogenesis and endochondral ossification

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The *toothless* (*tl*) mutation in the rat is a naturally occurring, autosomal recessive mutation resulting in a profound deficiency of bone-resorbing osteoclasts and peritoneal macrophages. The failure to resorb bone produces severe, unrelenting osteopetrosis, with a highly sclerotic skeleton, lack of marrow spaces, failure of tooth eruption, and other pathologies. Injections of CSF-1 improve some, but not all, of these. In this report we have used polymorphism mapping, sequencing, and expression studies to identify the genetic lesion in the *tl* rat. We found a 10-base insertion near the beginning of the open reading of the *Csf1* gene that yields a truncated, nonfunctional protein and an early stop codon, thus rendering the *tl* rat CSF-1^{null}. All mutants were homozygous for the mutation and all carriers were heterozygous. No CSF-1 transcripts were identified in rat mRNA that would avoid the mutation via alternative splicing. The biology and actions of CSF-1 have been elucidated by many studies that use another naturally occurring mutation, the *op* mouse, in which a single base insertion also disrupts the reading frame. The *op* mouse has milder osteoclastopenia and osteopetrosis than the *tl* rat and recovers spontaneously over the first few months of life. Thus, the *tl* rat provides a second model in which the functions of CSF-1 can be studied. Understanding the similarities and differences in the phenotypes of these two models will be important to advancing our knowledge of the many actions of CSF-1.

Osteoclasts, multinucleated cells that resorb bone, differentiate via fusion of mononuclear precursors of the monocyte/macrophage lineage, in large part under the local control of factors secreted by bone-forming osteoblasts (1). Insights into factors that regulate the formation and activation of osteoclasts have come from naturally occurring mutations and genetic manipulations that cause osteopetrosis, a condition in which defective bone resorption leads to a sclerotic skeleton (2). One such factor is the cytokine CSF-1 (M-CSF), originally identified as a monocyte-macrophage colony-stimulating factor (3). Identification of a frameshift mutation in the *Csf1* gene of the *osteopetrosis* (*op*) mouse was a major step in understanding osteoclast ontogeny (4). (The *op* mutation in the mouse, which affects an osteoblast signal, is not to be confused with the *op* mutation in the rat, a severe, naturally occurring osteopetrotic mutation that affects osteoclast function.) Since then, many genes have been identified that do double-duty in the immune and skeletal systems. Tumor necrosis factor superfamily member 11 [TNFSF11, also called TRANCE (5), RANKL (6), ODF (7), and OPGL (8)], its receptor (RANK), and the receptor-associated intracellular signal initiator (TRAF6) are essential for lymph node organogenesis, maintaining antigen presenting dendritic cells, and formation of osteoclasts (refs. 9–12; reviewed in ref. 13). The transcription factor PU.1 functions in osteoclast differentiation and activation and in myeloid cells and B lymphocytes (14); and NF-κB, originally described as regulating transcription of Ig light chain genes, is likewise necessary for osteoclastogenesis (15).

Phenotypes of osteopetrotic mutations vary widely, depending on where bone resorption is intercepted. Most of the proteins listed above act in the osteoclast or its precursors; however, two key factors that promote osteoclast differentiation and activation *in vivo*, CSF-1 and TNFSF11, are supplied by osteoblasts. These two are also necessary and sufficient to induce mononuclear precursors to fuse and differentiate into active, bone resorbing cells *in vitro* (16–18). One osteopetrotic mutation in the rat, *toothless* (*tl*), is due to the loss of such an osteoblast-derived, osteoclast-inducing signal, as demonstrated both *in vivo* and *in vitro*. In coculture experiments, *tl* osteoblasts cannot activate normal osteoclasts (19, 20), and the defect is not cell autonomous for the osteoclast lineage, because transplanting normal bone marrow or spleen-derived osteoclast precursors fails to improve *tl* rats (21). The *tl* rat is a naturally occurring, autosomal recessive mutation identified in 1974 (22). It exhibits severe, unremitting osteoclastopenia and a host of other pathologies. If phenotype is used as a guide to select candidate genes, the skeletal phenotype of *tl* rats resembles TNFSF11^{null} mice more than it does *op* (CSF-1 frame-shifted) mice. *op* mice have milder osteopetrosis and osteoclastopenia and recover in the first few postnatal months (23). TNFSF11^{null} mice and *tl* rats also share severe and progressive growth plate chondrodystrophy not seen in most other osteopetrotic mutations (9, 10, 24, 25). Despite the striking phenotypic similarities between *tl* rats and TNFSF11^{null} mice, we recently showed that this was not the locus of the *tl* mutation (26). We therefore undertook investigations to map the *tl* locus by positional cloning using polymorphic markers in the rat genome. Mapping and sequencing results revealed the presence of a CSF-1 frameshift mutation in the *tl* rat, thus rendering it a *Csf1*^{null} mutant.

Materials and Methods

Animals. *tl* mutants were obtained from the inbred colony maintained at the University of Massachusetts Medical School. The

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Abbreviations: BN, Brown Norway; TNFSF11, tumor necrosis factor superfamily member 11.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF515736).

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colony, which is on the Fischer background, was rederived in 1997 and has been kept under SPF conditions. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, and approved by the Institutional Animal Care and Use Committee. Female breeders of the Brown Norway (BN)/SSN strain were from Harlan Breeders (Indianapolis). Preliminary tests showed that many microsatellite markers are informative between the BN/SSN and *tl* strains. Diagnosis of osteopetrosis was done by neonatal x-ray (27), which shows the mutants to lack marrow cavities. Mature *tl* males (i.e., homozygous mutants) were bred with BN/SSN females to produce the obligate heterozygous F1 generation. F1 animals were then intercrossed to produce the F2 generation.

Mapping the *tl* Mutation. Genomic DNA was obtained from tail biopsies of 11 F2 mutants and used to map the chromosomal region carrying the *tl* mutation. Markers, from the Rat MapPairs screening set (Research Genetics, Huntsville, AL), were analyzed by PCR using radioactively labeled primers. After electrophoresis on polyacrylamide gels, PCR products were visualized by autoradiography. Additional markers were selected from the map based on the MIT (SHRSP × BN) F2 cross (www.rgd.mcw.edu/).

RT-PCR and Cloning of Normal and *tl* Rat CSF-1 cDNA. Total RNA was isolated from rat tissues by using TRIzol reagent according to the manufacturer's instructions (GIBCO/BRL). cDNA was synthesized by reverse transcription of 5 μg of total RNA with an oligo(dT) primer (SuperScript, GIBCO/BRL). cDNA products were used as templates for PCR amplification with primers flanking different regions of the ORF based on the published rat cDNA (ref. 28; accession no. NM_023981), and the PCR products were cloned into the TOPO 2.1 vector (Invitrogen) and sequenced as described (29).

Characterization of Normal Rat CSF-1 Genomic DNA. PCR primers sets were designed based on a published rat CSF-1 sequence (ref. 28; accession no. NM_023981) that encompassed the entire ORF. PCR products were purified with the Rapid PCR system (CLONTECH) and sequenced using Big Dye Terminator Cycle Sequencing (PE Applied Biosystems). Fragments were analyzed on an ABI 3100 automated sequencer. During the course of these investigations, our sequence was found to differ at various positions from the database sequence (see *Results*). We found that the previously reported sequence for rat CSF-1 is 100% identical to the mouse sequence and is thus a misidentified mouse cDNA sequence. The rat *Csf1* sequence was determined both on cDNA and genomic DNA samples. PCR reactions using primers that bridge introns were performed to analyze the exon/intron structure of the rat *Csf1* gene.

Identification of the *tl* Mutation. Direct sequence analysis was performed on both genomic DNA and RT-PCR products from *tl* rats. Once the mutation was identified, we determined the genotype of animals by using PCR primers that flank the mutation. PCR conditions: forward primer, 5′-GTTTGCCTCG-GTGCTCTCGG-3′; reverse primer, 5′-GAAGAAGGGCAG-CGCCCCG-3′; anneal at 60°C, 50 s; elongate at 72°C, 90 s; denature at 95°C, 50 s; 30 cycles. This results in a PCR product of ≈200 bp, permitting separation on polyacrylamide gels of wild-type fragments from mutants carrying the 10-base *tl* duplication.

Expression Analysis of Rat *Csf1* Gene on a cDNA Panel. Expression patterns and alternatively spliced variants of CSF-1 mRNA were evaluated with a rat Multiple Tissue cDNA panel (CLONTECH). PCR was performed on cDNA samples to amplify either the entire ORF, or just sequence from exons 1–3. Because of the naturally low levels of CSF-1 transcripts, the amplified products were separated on agarose gels, blotted, and probed with ^{32}P-labeled rat CSF-1 cDNA.

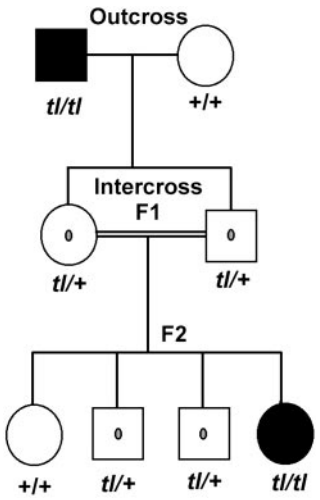


Fig. 1. Breeding scheme. Filled symbols, mutants; open symbols, wild type; spotted symbols, heterozygous mutants. Cosegregation of polymorphic markers and the mutation was examined in F2 animals.

Histology. Tibiae were dissected from *tl* rats, *op* mice, and normal littermates at 4 weeks of age, and processed for histochemical detection of the osteoclast enzyme tartrate-resistant acid phosphatase (TRAP) as described (30).

Results

Breeding Confirms *tl* Is a Single Gene Mutation. To map the *tl* locus, we outcrossed the inbred *tl* strain with the inbred strain Brown Norway/SSN. The latter was selected because it is genetically distinct from Fischer, resulting in a high percentage of difference in polymorphic markers (data not shown). Fig. 1 shows the breeding scheme. Mutant males, which are able to reach sexual maturity when husbanded under SPF conditions and fed a soft diet, were bred with BN/SSN females to produce the obligate heterozygote F1 generation. Crossing F1 pairs yielded the F2 generation, in which the *tl* phenotype occurred with the expected Mendelian frequency of about 1/4. The radiographs in Fig. 2 show that the *tl* phenotype bred true in this background, consistent with its being the product of a single mutation and not a strain-specific, multigene effect. A lack of marrow spaces in long bones was evident at birth. Tooth eruption, which requires

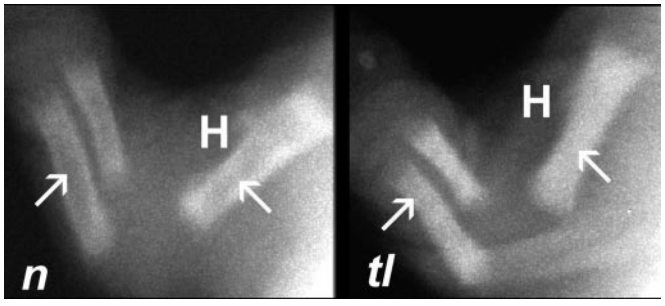


Fig. 2. *tl* phenotype breeds true in BN/SSN strain background. Radiographs of long bones of a newborn normal rat (*n*) and a mutant F2 generation littermate (*tl*) show high bone density and lack of marrow spaces in the mutant (arrows). H, humerus.

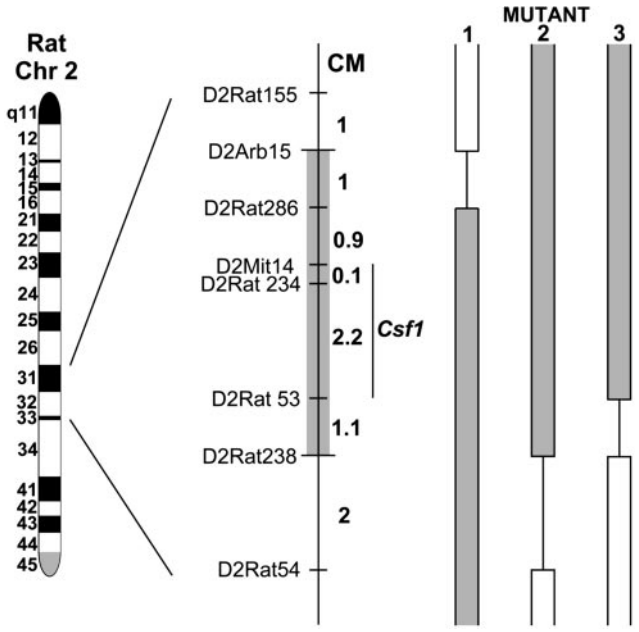


Fig. 3. Ideogram of rat chromosome 2 and genetic map of markers used to delineate candidate region. The candidate region is shown as a gray bar on the first vertical line. Key recombinants are given on the other vertical lines. Gray bars represent chromosomal regions that contain the disease gene, and white bars indicate regions that recombined and therefore cannot contain the disease gene. The lines between represent uninformative regions. The gray regions include *Csf1*.

osteoclast excavation of eruption pathways through the jaws, did not occur; and the osteopetrotic phenotype persisted for the full 2 months that we kept outcrossed mutants (not shown).

Genetic Localization of the *tl* Mutation. We performed genetic analysis on 11 mutant *tl*/*tl* F2 animals. Markers were selected from the rat chromosome 2 region syntenic with human chromosome 1p21, where the human *Csf1* gene is located (Online Mendelian Inheritance in Man, www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = OMIM). Homozygosity for the Fischer allele in all affected animals was found for four of the markers analyzed (D2Rat286, D2Mit14, D2Rat234, and D2Rat53; Fig. 3). Because these are completely linked with the disease, a maximum LOD score of +6.6 is obtained at recombination fraction 0. On the proximal side the disease recombines with D2Arb15 in mutant 1, whereas on the distal side a recombination event is detected with both D2Rat238 and D2Rat54 in mutant 3 and with D2Rat54 in mutant 2. This refined the candidate region to 5.3 cM, flanked by the markers D2Arb15 and D2Rat238 (Fig. 3). Based on synteny between mouse, human, and rat (Rat Genome Database), the rat *Csf1* gene is located on rat chromosome 2q34–45, between markers D2Mit14 and D2Rat 53, precisely within the delineated candidate region (Fig. 3).

Normal Rat CSF-1 Sequence and Gene Structure. Sequencing rat CSF-1 genomic or cDNA from both *tl*/*tl* and normal rats revealed a large number of differences from the published rat CSF-1 cDNA sequence (ref. 28; NM_023981). When performing alignments of the published nucleotide and amino acid sequences of normal rat and mouse (e.g., accession no. NM_007778.1) CSF-1, 100% identity was found. This 100% identity is in fact shown in a rat vs. mouse multiple alignment in the original publication (28). Thus, we conclude that the published sequence is actually a misidentified mouse CSF-1 cDNA.

The sequence reported here for rat CSF-1 was obtained from both genomic DNA and cDNA. A complete ORF of 1701 bp as well as partial 5′ and 3′ UTR sequences have been deposited in GenBank (accession no. AF515736). CSF-1 is highly conserved between rat, mouse, and human (see Fig. 4). Identity of rat and mouse nucleotide sequence is 91%, 86% for amino acids, with an additional 3% conservative substitutions and two small gaps that preserve the reading frame. The rat sequence, however, has a unique feature: a 78-nt direct repeat after position 1380 of the ORF. This maintains the reading frame, but inserts a

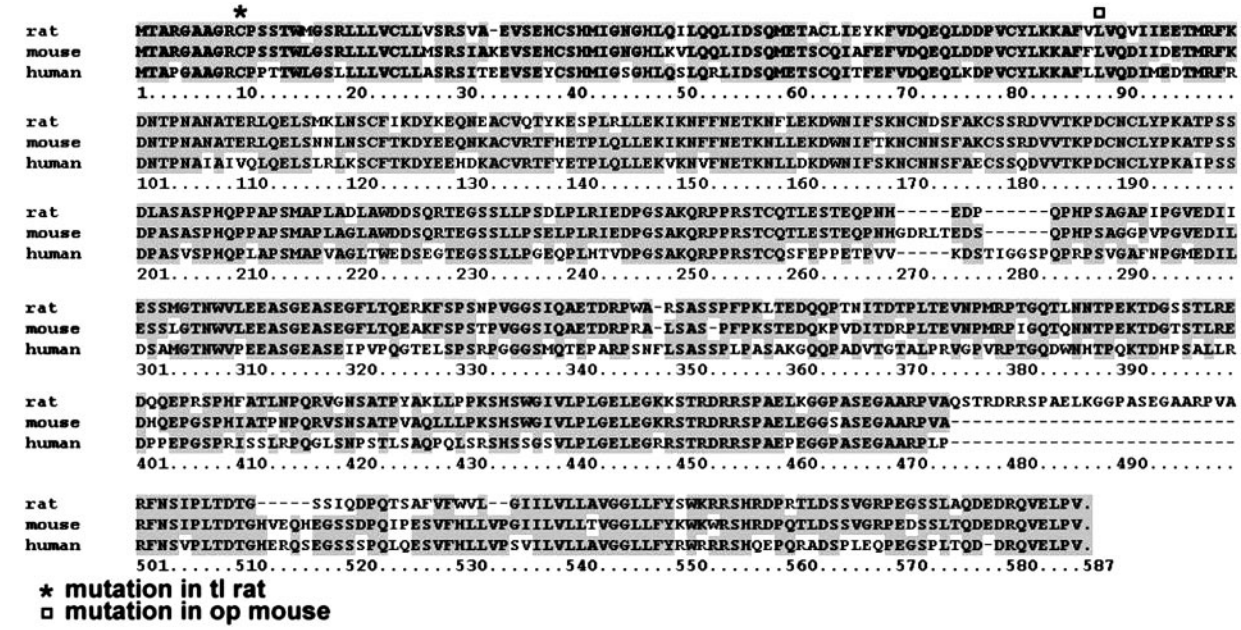
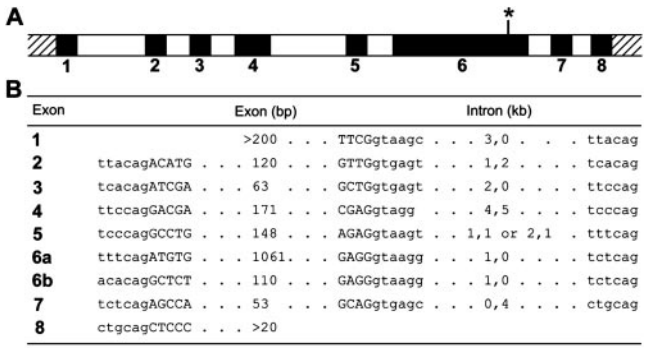


Fig. 4. Alignment of amino acid sequences of rat, mouse, and human CSF-1 full-length ORF show high conservation. The insertion in the rat from position 476–501 of the consensus is due to a nucleotide duplication unique to the rat. Excluding this stretch, there is >93% identity among the species, plus a number of conservative substitutions. The points of *tl* rat and *op* mouse frameshifting insertions are indicated. The normal rat cDNA and amino acid sequences have been deposited in GenBank (accession no. AF515736).



Exon	Exon (bp)	Intron (kb)
1	>200 . . .	TTCGgtaagc . . . 3,0 . . . ttacag
2	ttacagACATG . . . 120 . . .	GTTGgtgagt . . . 1,2 . . . . tcacag
3	tcacagATCGA . . . 63 . . .	GCTGgtgagt . . . 2,0 . . . . ttccag
4	ttccagGACGA . . . 171 . . .	CGAGgtagg . . . 4,5 . . . . tcccag
5	tcccagGCCTG . . . 148 . . .	AGAGgtaagt . . 1,1 or 2,1 . tttcag
6a	tttcagATGTG . . . 1061. . .	GAGGgtaagg . . . 1,0 . . . . tctcag
6b	acacagGCTCT . . . 110 . . .	GAGGgtaagg . . . 1,0 . . . . tctcag
7	tctcagAGCCA . . . 53 . . .	GCAGgtgagc . . . 0,4 . . . . ctgcag
8	ctgcagCTCCC . . . >20	

Fig. 5. Organization of rat *Csf1* gene. (*A*) The ORF of the gene is dispersed in 8 exons, shown with black boxes. 5′ and 3′ UTRs are shown with stripes. The alternative splice acceptor site in exon 6 is marked (asterisk). (*B*) Sequences of the intron/exon boundaries of the rat *Csf1* gene and the length of each. Uppercase letters, exon sequences; lowercase letters, intron sequences.

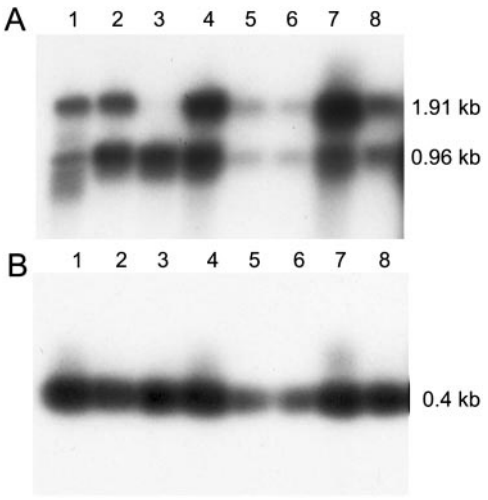


Fig. 7. Alternative splicing of rat CSF-1 mRNA reveals no skipping of *tl* mutation-containing region. CSF-1 cDNAs were amplified from rat cDNA, separated in an agarose gel, blotted, and probed with ^{32}P-labeled rat CSF-1 cDNA. Lanes 1–8 represent skeletal muscle, liver, kidney, lung, brain, testis, pancreas, and heart, respectively. (*A*) RT-PCR product encompassing the complete coding sequence of rat CSF-1. Two alternative transcripts of 0.96 and 1.91 kb, respectively, can be distinguished. The shorter fragment can be explained by alternative splicing deletion of the major part of exon 6, as has been described in mouse and human. (*B*) PCR product containing the first 3 exons of rat CSF-1 cDNA. Only one transcript can be distinguished, indicating no alternative splicing in the 5′ part of the transcript that could bypass the *tl* mutation.

26-aa repeat following Ala-460 in rat (Ala-474 of consensus). Structural/functional implications of the repeat remain to be determined.

We also determined the intron/exon structure of the rat *Csf1*. Fig. 5*A* shows the results of this analysis. The organization of the rat gene is similar to mouse and human. The ORF of rat *Csf1* is divided into 8 exons split by 7 introns, spanning a genomic region of ≈15 kb. The first exon contains the 5′ UTR, plus the first 13 aa of the leader sequence. The remaining 19 aa of the leader sequence are in exon 2. The translation stop signal (TAG) is in exon 8. The sequence of exon/intron boundaries and sizes of exons and introns are given in Fig. 5*B*.

Identification of the *tl* Mutation. Sequence analyses of *tl* rat CSF-1, both genomic DNA and cDNA, showed a 10-bp duplication in exon 1 (Fig. 6*A*). This 10-nt sequence occurs twice in the normal rat, but in the *tl* mutation it occurs three times in tandem. This results in a frameshift mutation after codon 9 (before the signal peptide), followed by an additional 32 unrelated amino acids and a stop codon, thus yielding an abnormal, highly truncated protein. As expected, the mutation was homozygous in all *tl*/*tl* animals tested.

Evaluation of Alternative Splicing in Rat CSF-1 Transcripts. To understand the severity of the *tl* phenotype and verify that it is due to the mutation in the amino-terminal portion of the encoded protein, we tested whether alternatively spliced CSF-1 transcripts were present in rat tissues (Fig. 7). A panel of rat tissue cDNAs was amplified using primers spanning either the entire CSF-1 ORF or just exons 1–3, and probed with CSF-1 cDNA. Two transcripts were detected when the entire coding region was amplified. Sequencing these indicated that they are consistent with the alternatively spliced exon 6 variants seen in mouse and human (refs. 31–34; Fig. 5). Only one band was seen for the first 3 exons, demonstrating that exon 1 is not bypassed in any alternatively spliced CSF-1 mRNA species, and therefore that the *tl* mutation prevents any functional CSF-1 from being made.

Greater Severity of Osteopetrosis in *tl* Rats Versus *op* Mice. Fig. 8 shows the virtually complete lack of osteoclasts in *tl* rats. In contrast, osteoclasts are present in *op* mice, increasing in number with age at some skeletal sites. TRAP-positive osteoclasts are normally highly abundant in the tibial metaphysis at 4 weeks postpartum. Although it can be seen that the osteoclast population in the *op* mouse is markedly deficient, it is nevertheless far greater than in the *tl* rat. This ability to produce osteoclasts

Fig. 6. The *tl* mutation. (*A*) Nucleotide and amino acid sequence of the *tl* CSF-1 mutation. The 10-nt repeat, underlined, commences in the third codon. It occurs twice as a single repeat in the normal sequence, but in the *tl* mutation it occurs three times. The impact on the protein is seen in the translations, below, in which the mutant sequence diverges 9 residues from the initiation Met, continues for 33 residues, and then reaches a stop codon (*). The signal sequence of the wild-type protein, double underlined, is absent from the *tl*. Only the first 126 bases of the ORFs are shown. (*B*) Genotyping with PCR primers that flank the mutation. After separating on a polyacrylamide gel, wild-type (+/+) fragments can be distinguished from mutants (*tl*/*tl*) carrying the 10-base *tl* duplication. Heterozygotes (*tl*/+) clearly show two bands corresponding to the mutant and wild-type alleles.

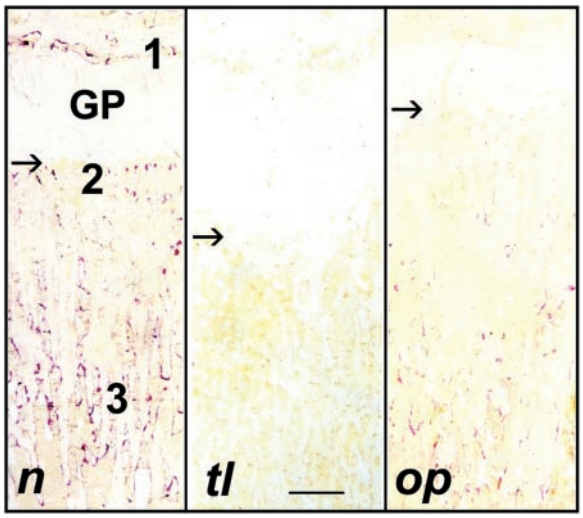


Fig. 8. Skeletal phenotype of *tl* rat compared with *op* mouse. Proximal tibiae from 4-week-old normal rat (*n*), *tl* rat (*tl*), and *op* mouse (*op*) stained histochemically for the osteoclast marker enzyme tartrate-resistant acid phosphatase (TRAP) (red). Sections are aligned along the top of the growth plate (GP). Arrows indicate lower margin of growth plate. Osteoclasts are evident in *n*, where remodeling is most active: 1, top of the GP; 2, bottom of the GP at chondroosseous junction; 3, near the ends of metaphyseal trabeculae. No TRAP-positive osteoclasts are seen in the CSF-1 mutant *tl* rat. In contrast, by 4 weeks, the *op* mouse has partially reestablished osteoclast populations in the metaphysis. (Scale bar, 310 μm.)

despite the *op* mouse frameshift mutation in CSF-1 has been difficult to explain, particularly with the increasing body of evidence showing that a combination of CSF-1 and TNFSF11 is necessary and sufficient for osteoclastogenesis *in vitro*. Fig. 8 underscores this question, given the much more severe osteoclastopenia in the CSF-1 mutant *tl* rat.

Discussion

Mapping, sequencing, and expression results described here define a single genetic defect in *tl* rats residing in the *csf1* gene. This finding is entirely consistent with all previous investigations of *tl* rats, including:

1. Colony stimulating and macrophage population studies (35) showed that postendotoxin *tl* rat serum lacks colony stimulating activity, consistent with what was seen in *op* mice (36).
2. CSF-1 injections (25, 35, 37–40). The beneficial effects of injections of CSF-1 in *tl* rats are well studied. They improve some, but not all, phenotypic defects. Most amenable to improvement are peritoneal macrophages (35), diaphyseal and metaphyseal osteoclast populations (35, 37, 38), thrombocytopenia (39), and tooth eruption (35, 41). Skeletal problems unresponsive to CSF-1 injections, in particular the metaphyseal sclerosis, growth plate chondrodystrophy, and long bone growth (25), likely involve pathways requiring local supply of CSF-1 in its matrix-associated or cell-bound forms, as was suggested for certain CSF-1-resistant defects in the *op* mouse (42, 43). Also, CSF-1 may be required *in utero* to establish some normal cell populations, and postpartum injections may simply come too late for benefit.
3. Stem cell transplants (21). Were the *tl* defect to reside in an osteoclast-specific gene, then transplanting normal mononuclear precursors should restore osteoclast function and cure the osteopetrosis. This does not happen in *tl* rats, and so is consistent with the gene affected being expressed by osteoblasts, as CSF-1 is known to be.
4. Bone cell cocultures (19, 20). Cultured normal osteoblasts, when stimulated by 1,25-dihydroxyvitamin D_3, secrete both CSF-1 and TNFSF11, inducing differentiation and activation of cocultured osteoclasts. *tl* osteoblasts are incapable of this. Because the *tl* mutation is not in TNFSF11 (26), this alone would make CSF-1 a strong candidate for the *tl* mutation.

Together, all these studies support the conclusion drawn here from our mapping, sequencing, and expression studies, that the frameshift mutation in CSF-1 is the underlying genetic defect in the *tl* rat.

The variety of phenotypic consequences of this single-gene mutation in *tl* rats implies a wide-ranging activity for the encoded protein. In addition to direct consequences of the failure of bone resorption due to a near-complete lack of osteoclasts, other manifestations are intriguing. *tl* rats exhibit abnormal angiogenesis at the chondroosseous junction (44); have a highly unusual, progressive chondrodystrophy in the growth plates of endochondral bones (24, 25); have disorganized actin stress fibers in osteoblasts, accompanied by mis-sorting of β-actin mRNA (45, 46); fail to switch collagen gene expression at sites of bone growth by both endochondral and intramembranous processes (47, 48); have retarded growth at these sites (25); and exhibit endocrine abnormalities in vitamin D levels and decreased levels of GH receptor and of IGF1 and its receptor (49–52).

High conservation of CSF-1 among human, rat, and mouse implies conserved functions; nevertheless, the spectrum of phenotypic consequences of the *tl* mutation is broader and more persistent than the other well studied CSF-1 loss-of-function mutant, the *op* mouse (*cf.* Fig. 8). Some compensatory mechanism may act in *op* mice that is absent from the rat model—perhaps species or background stock differences. Hume and Favot (53) proposed an alternative spicing event that could bypass the exon carrying the *op* mutation, but whether the translated product of the resulting message could function has never been demonstrated. Alternatively, recent studies show a potentially important role for VEGF in recovery of the *op* mouse (54). Exploration of these and other possible mechanisms will deepen our understanding of the biology and actions of CSF-1.

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