

Colony-stimulating factor-1 (CSF-1) rescues osteoblast attachment, survival and sorting of β -actin mRNA in the toothless (*tl*-osteopetrotic) mutation in the rat

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ABSTRACT We have shown that in the osteopetrotic rat mutation toothless (*tl*) osteoblasts are absent from older bone surfaces in mutants and that mutant osteoblasts *in vivo* lack the prominent stress fiber bundles polarized along bone surfaces in osteoblasts from normal littermates. Our recent data demonstrate that in normal osteoblasts *in vitro* β - and γ -actin mRNAs have different, characteristic intracellular distributions and that *tl* (mutant) osteoblasts fail to differentially sort these mRNAs. Because bone resorption and formation are highly interdependent and injections of CSF-1, a growth factor, increase bone resorption and growth in *tl* rats, we examined the effects of CSF-1 treatment on osteoblast survival and ultrastructure *in vivo* and ability to sort actin mRNAs *in vitro*. Neonatal CSF-1 treatment of mutants restores osteoblasts on older bone surfaces, normalizes the intracellular distribution of stress fibers in osteoblasts *in vivo* and promotes normal sorting of β -actin mRNA in mutant osteoblasts *in vitro* without normalizing γ -actin distribution. These data suggest the β - and γ -actin mRNAs in osteoblasts are sorted by different mechanisms and that the differential sorting of β -actin mRNA is related to the characteristic polarization of stress fibers in osteoblasts and their survival on bone surfaces. This experimental system can be used to explore the relationships and regulation of these aspects of cell and tissue biology.

KEY WORDS: *cell attachment, actin isoforms, stress fibers, osteoblast*

Introduction

The differentiation and survival of normal cells depends on appropriate attachment to adjacent cells and/or extracellular matrices (Frisch and Ruoslahti, 1997). Participating in these interactions are stress fibers, linear arrays of microfilament bundles (Byers *et al.*, 1984). Studies have shown that stress fibers are composed of actin (Yahara *et al.*, 1982; BurrIDGE *et al.*, 1988) and are associated with alpha-actinin and vinculin at sites where they attach to the cell membrane (Geiger, 1982). Watanabe *et al.* (1993a,b) have identified stress fibers in osteoblasts *in vivo* and showed that these filaments contained actin and alpha-actinin where the microfilament bundles are bound to the cell membrane. Stress fibers connect adhesion plaques (Byers and Fujiwara, 1982; BurrIDGE and Connell, 1983; Turner and BurrIDGE, 1991) and provide strength between adhesion points for attachments to the extracellular matrix.

The cytoskeletal actin isoforms, β - and γ -, are ubiquitous components of the microfilament system. That these two actin isoforms may serve fundamentally different roles is suggested by significant differences by β/γ ratios between tissue types (Otey *et al.*, 1987) and by the observations that actin mRNAs are differentially sorted (Hill and Gunning, 1993; Kislauskis *et al.*, 1993). Just how expression and/or sorting of β - and γ -actin isoforms coordinate cell structure and function remains unclear. We have recently reported that β and γ -actin mRNAs are differentially sorted in normal osteoblasts (Watanabe *et al.*, 1998) and that these cells have prominent stress fiber bundles that occur alongside 75% of the cell surface next to bone matrix (Watanabe *et al.*, 1997). That

Abbreviations used in this paper: CSF-1, colony-stimulating factor-1; *tl*, toothless mutation in the rat; TEM, transmission electron microscopy; MEM, minimal essential medium.

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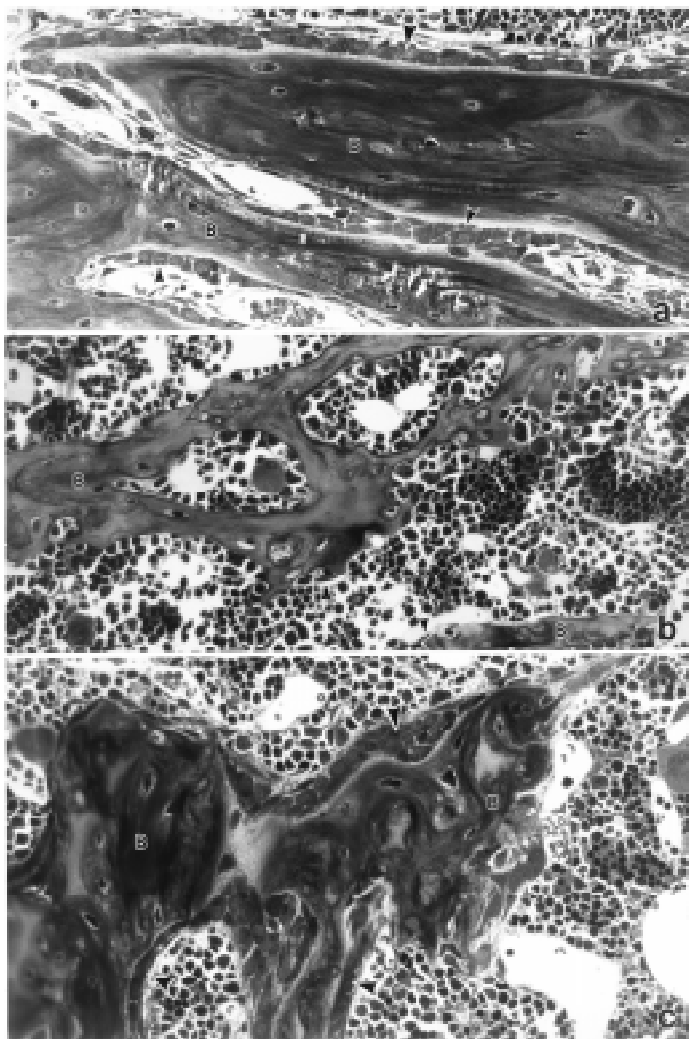


Fig. 1. Representative photomicrographs of the deep proximal tibial metaphysis of untreated normal (a) and mutant (b) rats and a mutant treated for three weeks with CSF-1 (c). Metaphyseal bone (B) in normal rats and in CSF-1-treated mutants is thick and its surfaces are covered by a layer of prominent osteoblasts (arrowheads). Bone (B) in untreated mutants is thin, is covered by a few bone lining cells (arrowheads) and commonly juxtaposed to bone marrow cells and stroma. Toluidine blue. X380.

these events are functionally related in osteoblasts is suggested by the observations that, in the skeletal mutation toothless (*tl*), in the rat, osteoblasts show ultrastructural features of premature apoptosis, are not present on many bone surfaces, have significantly shorter stress fibers *in vivo* (Watanabe *et al.*, 1997) and fail to sort both β - and γ -actin mRNAs in primary cultures (Watanabe *et al.*, 1998). These data suggest that the paucity of osteoblasts in older parts of the mutant skeleton may be related to the impaired ability of *tl* osteoblasts to properly localize actin mRNAs, potentially resulting in random actin synthesis, assembly and attachment of stress fibers to extracellular matrices. Based on the recent demonstration that cell attachment area is crucial to survival (Chen *et al.*, 1997), the early demise of osteoblasts in *tl* rats may be caused by this lack of attachment.

Based on an emerging role of signal transduction, specifically that mediated by tyrosine kinases, in the production and function of actin (Ridley and Hall, 1994) and in actin mRNA localization (Latham *et al.*, 1994), we have extended our earlier studies of stress fibers and actin mRNA localization in osteoblasts of *tl* rats by studying the effects of treatment with colony-stimulating factor-1 (CSF-1). CSF-1 is a cytokine whose receptor, *c-fms*, a tyrosine kinase, is present on osteoclasts and their mononuclear precursors but not osteoblasts (Hofstetter *et al.*, 1992). However, the activities of osteoclasts and osteoblasts are reciprocally interdependent (Marks and Popoff, 1988) and treatment of *tl* rats with CSF-1 restores certain aspects of gene expression in osteoblasts and reduces skeletal sclerosis (Marks *et al.*, 1993, 1992; Wisner-Lynch *et al.*, 1995). Here we evaluated the effects of CSF-1 treatment on the actin and stress fiber abnormalities previously described in *tl* rats (Watanabe *et al.*, 1997, 1998) to test the hypothesis that the *tl* phenotype is the result of an interrupted CSF-1 signal transduction pathway that is critical for differential sorting of actin mRNAs and for the normal assembly and membrane association of stress fibers to anchor osteoblasts within their extracellular matrices.

Results

The effects of CSF-1 treatment on osteoblasts are shown in Figure 1. In normal rats (Fig. 1a) bone surfaces are covered by large osteoblasts, occasionally in layers. Bone surfaces in untreated mutants, on the other hand, have few osteoblasts and are often in juxtaposition to the hemopoietic stroma (Fig. 1b). CSF-1 treatment of mutants restores osteoblasts on bone surfaces (Fig. 1c). The extent to which osteoblasts cover bone surfaces in these three groups of rats is shown in Figure 2. In normal rats, osteoblasts occupy 88 (± 2) percent of the bone surfaces in the tibial metaphysis. The remaining bone surface was occupied by osteoclasts in these growing bones. In untreated *tl* rats osteoblasts can be found on 30 (± 17) percent of these surfaces, a significant ($p \leq 0.02$) decrease from that in normal littermates. In untreated *tl* rats osteoblasts occupied more of younger bone surfaces (44 ± 8 percent when measured within 0.5 μm of the growth plate) than older bone surfaces (17 ± 9 percent when measured greater than 1.5 μm from the growth plate). This significant decline ($p \leq 0.05$) in osteoblasts on bone surfaces as they age (i.e., move away from the growth plate) suggests accelerated cell death in untreated mutants. CSF-1 treatment of *tl* rats restores osteoblasts on bone surfaces, to 90 (± 5) percent.

Ultrastructural examination of osteoblasts showed (Fig. 3) that after CSF-1 treatment mutant osteoblasts exhibited stress fiber bundles along the cell surface next to bone (Fig. 3e,f). These long stress fibers resembled those seen in normal osteoblasts (Fig. 3a,b) not the short segmented fibers which could occasionally be found in osteoblasts of untreated mutants (Fig. 3c,d). To clarify the effects of CSF-1 treatment on stress fibers we calculated the percentage of the osteoblast cell surface next to bone matrix in which well-developed stress fiber bundles could be identified (Fig. 4). Measurements from untreated normal and mutant rats agreed with our earlier work (Watanabe *et al.*, 1997). In normal osteoblasts, stress fibers were found along 75% of the cell surface next to the unmineralized bone matrix, osteoid. In untreated mutants, stress fibers were short and were present along less than 15% of

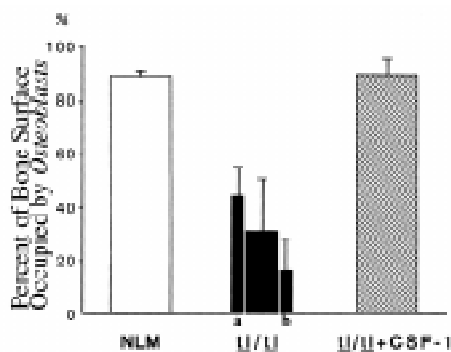


Fig. 2. Osteoblasts on bone surfaces in the proximal 2.5 μ m of tibial metaphysis of normal (NLM), untreated *tl* (*tl/tl*) and CSF-1-treated *tl* (*tl/tl* + CSF-1) rats, expressed as a percentage of the bone surface (+ S.D.). Shoulders "a" and "b" above *tl/tl* indicate values within 0.5 μ m and greater than 1.5 μ m from the growth plate, respectively.

this surface of osteoblasts. As reported earlier (Watanabe *et al.*, 1997) stress fibers could be identified in less than half of mutant osteoblasts. When present, stress fibers were short (occupying less than 25% of the cell surface length) or were not next to the cell surface (data not shown). CSF-1 treatment of *tl* rats restored stress fiber length in osteoblasts (Fig. 4). In summary, stress fibers in osteoblasts of untreated *tl* rats were significantly shorter ($p \leq 0.01$) than in untreated normal rats or CSF-1-treated mutants and their relative lengths in the latter two groups did not differ significantly.

Finally, we evaluated the effect of CSF-1 treatment for 4 days *in vivo* on the sorting of β - and γ -actin mRNA isoforms in primary cultures of *tl* osteoblasts. These data are shown in Figure 5 by comparisons of the phenotypic localizations of these signals in osteoblasts. The distributions we observed for both β - and γ -actin mRNAs in cultures of osteoblasts from untreated normal and mutant rats did not differ from the more extensive evaluation we published earlier (Watanabe *et al.*, 1998) and we have included the latter data in these figures. In normal osteoblasts β -actin mRNA was located primarily in cell processes or on one side of the nucleus and γ -actin mRNA in a perinuclear location with

very few cells exhibiting either a diffuse distribution or no signal (Fig. 5a,b). This significant differential sorting of actin mRNA isoforms was not seen in osteoblasts from untreated mutants, in which the predominant phenotype was a diffuse pattern of expression (Fig. 5a,b) (Watanabe *et al.*, 1998). Short-term neonatal treatment of *tl* rats with CSF-1 before isolating calvarial osteoblasts for primary culture restored the normal expression pattern for β -actin mRNA (Fig. 5a) but had no effect on the distribution of γ -actin mRNA (Fig. 5b). CSF-1 treatment had no effect on the mRNA levels of β -actin or γ -actin in osteoblast cultures (data not shown). Thus, the change in β -actin mRNA localization was not due to a change in β -actin expression but likely to specific mRNA transport itself.

Discussion

These data show that CSF-1 treatment of *tl* rats *in vivo* restores osteoblasts on bone surfaces, normalizes the appearance and distribution of stress fibers *in vivo*, and normalizes β -actin mRNA, not γ -actin mRNA, sorting by osteoblasts *in vitro*. Furthermore, these data suggest a genetic link between actin mRNA localization, stress fiber dynamics and localization, and osteoblast adhesion and survival. Given the emerging significance of tyrosine

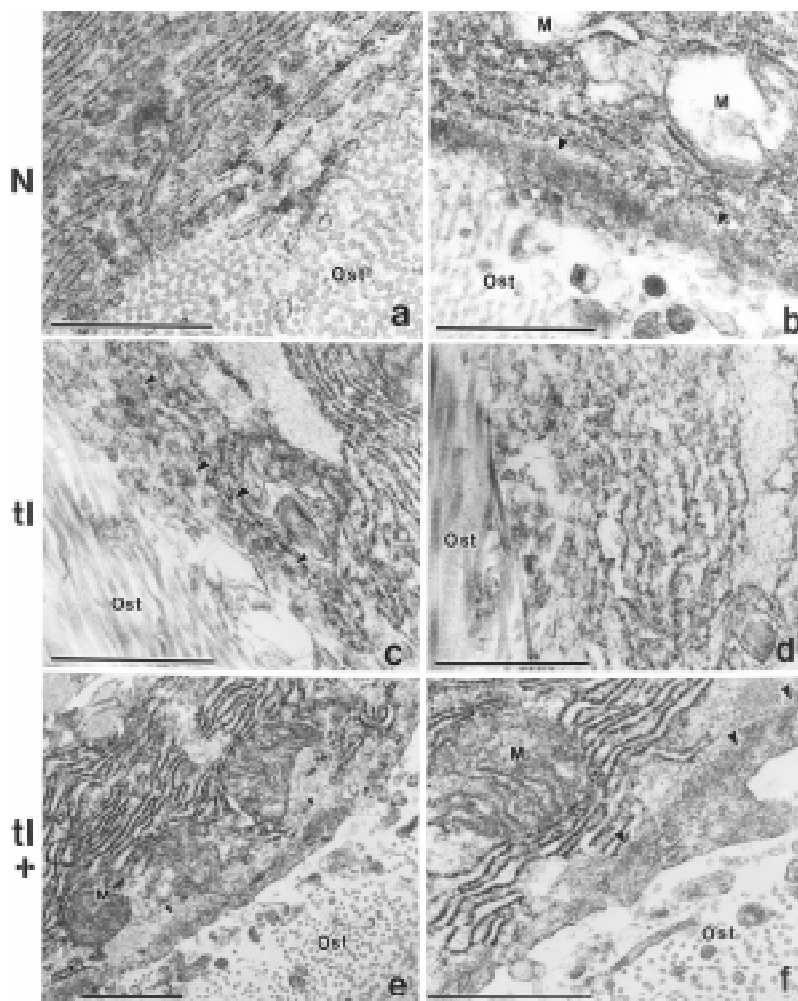


Fig. 3. Representative transmission electron micrographs of interfaces between osteoblasts and unmineralized bone matrix, osteoid (ost) in the proximal tibial metaphysis of normal (a,b), untreated *tl* (c,d) and CSF-1-treated *tl* (e,f) rats perfused, fixed and stained to reveal microfilament bundles in the vicinal cytoplasm (b-f). Without Triton-X perfusion (a) stress fibers are obscured by the high electron density of the cytoplasm. Perfusion and fixation that includes Triton-X (b) reveals thick bundles of stress fibers (arrowheads) in normal osteoblasts just deep to the cell membrane overlying osteoid (ost). In untreated *tl* mutants (c and d), stress fibers are more difficult to identify and when present (c) are in short, thin bundles. CSF-1 treatment of mutants (e and f) restores stress fibers in osteoblasts. M, mitochondrion; Bar, 1 μ m in a, b, c and d. f, X36,000; e, X23,000.

kinases in sorting of actin mRNAs (Latham *et al.*, 1994), we interpret these data to mean that: 1) CSF-1 and its receptor, *c-fms*, a tyrosine kinase, in osteoclasts cause release of a molecule(s) that binds to an unidentified receptor on osteoblasts to promote cell attachment, stress fiber formation and sorting of β -actin mRNA, 2) that survival of osteoblasts on bone surfaces is correlated with β -actin mRNA sorting and the formation of specifically positioned stress fibers and 3) that the sorting of γ -actin mRNA in osteoblasts is either not related to osteoblast survival or that its normal function can be accomplished with less than 50% of its mRNA sorted to a perinuclear position, as is the situation in *tl* osteoblasts.

The autosomal recessive mutation responsible for the *tl* phenotype in the rat has not been mapped. While this mutation bears some phenotypic similarities to the *op* mutation in the mouse (a natural CSF-1 knock-out, Yoshida *et al.*, 1990), analyses of CSF-1 cDNA sequence, transcripts and circulating levels of CSF-1 in *tl* rats show no abnormalities (Stanley and Marks, unpublished data). However, the ability of CSF-1 injections to induce osteoclast differentiation and function and to reduce skeletal sclerosis in *tl* rats (Marks *et al.*, 1992, 1993) suggests that the genetic defect in this mutation is in a growth factor with some overlapping functions with CSF-1. It has long been known that reciprocal factors from osteoblasts and osteoclasts couple and coordinate their activities in the skeleton (Marks and Popoff, 1988). The appearance of osteoclasts and the restoration of osteoblasts in *tl* rats treated with CSF-1 offers a system in which the connections between these effects can be explored.

Because *c-fms*, the receptor for CSF-1, is found on cells of the mononuclear phagocyte lineage, not osteoblasts (Hofstetter *et*

al., 1992) the effects of CSF-1 on *tl* osteoblasts in this study are indirect (Felix *et al.*, 1994). It is likely that following CSF-1 treatment mononuclear cells, including osteoclast precursors, are stimulated and produce factors that modulate osteoblast differentiation and function. These factors, in turn, somehow affect actin mRNA localization, stress fiber assembly, and cell attachment and survival of osteoblasts. Thus, the indirect effects of CSF-1 on osteoblasts in the *tl* mutation represent an *in vivo* model system to study the relationships between actin mRNA localization, stress fiber assembly, cell attachment and survival in skeletal development and pathology. What we understand about mRNA sorting, stress fiber formation and cell survival has been derived mostly from *in vitro* studies which speak to no more than two of these processes in isolation. The ability to study them together in the complicated dynamics of an integrated living system has definite advantages. This will permit exploration of significant questions for both basic and clinical scientists. Basic questions include sequence of events that initiate and sustain cell attachment and mRNA sorting and the relative contributions of β - and γ -actin sorting in biological processes. Clinical questions include whether osteoblast differentiation and recruitment can be monitored and modified *in vivo* to regenerate bone lost in local or generalized osteoporotic conditions by selectively increasing bone formation at these sites.

Focal adhesion complexes provide sites of attachment and a microenvironment for the continuous production of protein components of the focal adhesion complex via mRNA localization (Chicurel *et al.*, 1999). Changes in signal transduction, involving tyrosine kinases, regulate β -actin mRNA localization in fibroblasts and muscle cells (Hill *et al.*, 1994; Latham *et al.*, 1994). Underlying the change in shape is a reorganization of the cytoskeleton to create a lattice for message localization (Bassell *et al.*, 1994; Bassell and Singer 1997). The small GTPases Rho, Rac, and CDC25 function as molecular switches of signal transduction pathways that connect cell surface receptors with the actin cytoskeleton (Hall, 1994). Rho activation, in particular, induces stress fiber assembly and focal adhesion complex formation (Ridley and Hall, 1992). However, the specific relationship between β -actin mRNA localization and cell attachment remains unclear. In motile cells, the distribution of β -actin mRNA localization is correlated with cell shape (Kislauskis *et al.*, 1993) and motility (Kislauskis *et al.*, 1997) *in vitro*. The osteoblast, though fibroblast-like, is a highly polarized cell *in vivo* (Marks and Hermey, 1996). Whether motile and polarized cells use similar patterns of β -actin localization *in vivo* remains to be established.

Based on the current understanding of the role of signal transduction in development of cell attachment, message localization and cell polarity in other developing systems and in isolated cells, we propose the following hypothesis for the effects of CSF-1 on osteoblasts observed in this study. Exogenous treatment with CSF-1 induces osteoclastogenesis and function in the *tl* rat (Marks *et al.*, 1992). Growth factors produced by osteoclasts then feedback to modulate osteoblast attachment and survival. Continuous delivery of CSF-1 is necessary to maintain osteoclast function (Marks *et al.*, 1993) and presumably osteoblast attachment. In our model, the integrity of the actin cytoskeleton is critical for osteoblast attachment, and stress fiber length and distribution are indicators of cell attachment. In the *tl*

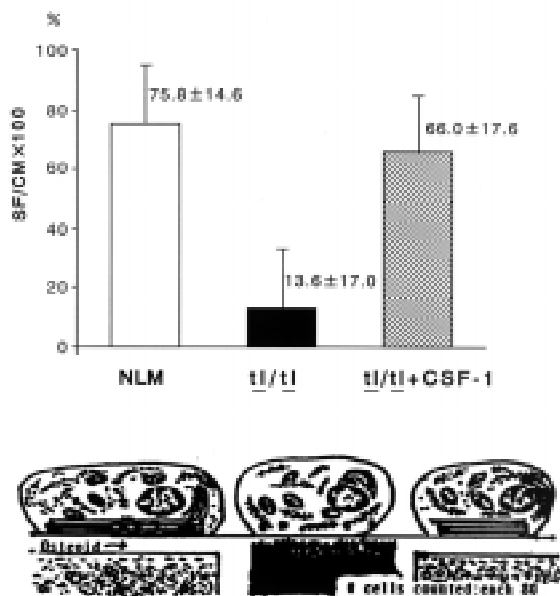


Fig. 4. Histogram of stress fiber (SF) length in osteoblasts, expressed as a percentage of the length of the cell membrane (CM) in contact with unmineralized bone matrix. NLM, normal littermate; *tl/tl*, toothless rat; *tl/tl*+CSF-1, CSF-1-treated toothless rat.

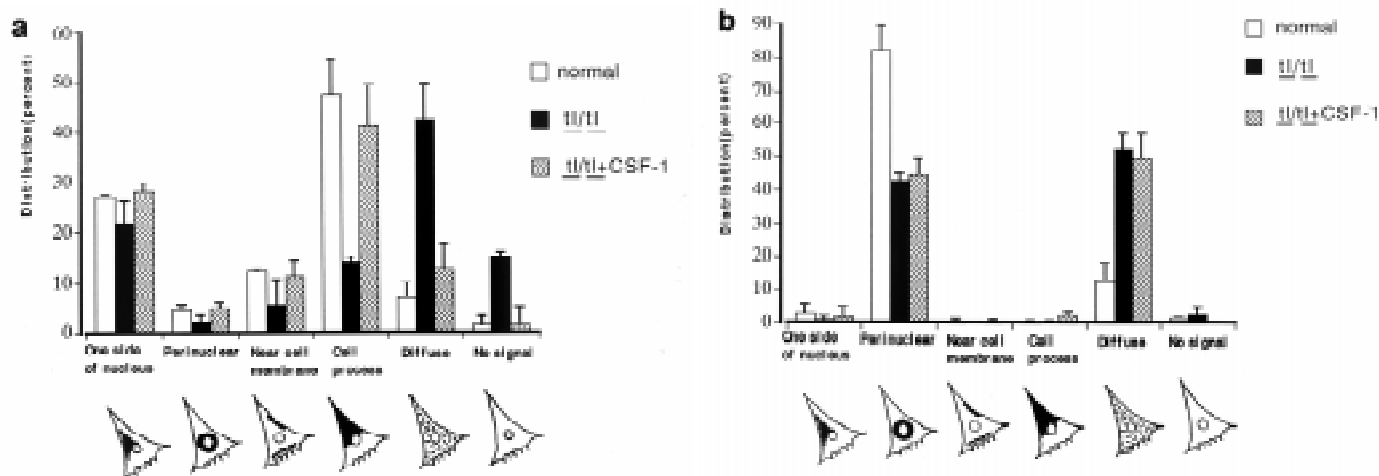


Fig. 5. Histograms of classifications of the intracellular localization of β -actin mRNA (a) and γ -actin mRNA (b) in osteoblast cultures from untreated normal and *tl/tl* rats and mutant littermates treated with CSF-1 for 4 days prior to cell isolation and culture. Data expressed as mean \pm S.D. for the phenotypes illustrated.

rat, a reduction in receptor-mediated signaling compromises cell attachment, stress fiber length, and β -actin mRNA localization. Each component of this hypothesis can be directly tested in this model.

Materials and Methods

Source and treatment of animals

Rats were obtained from breeding colonies at the University of Massachusetts Medical School, USA. Mutant animals (*tl/tl*) were identified radiographically at birth under hypothermic anesthesia by the failure to develop marrow cavities in long bones. Normal homozygotes (+/+) and heterozygotes (+/*tl*) are distinguishable only by breeding, and normal littermates included both these genotypes. Animals were maintained and used in accordance with recommendations in the Guide for Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publication NIH 86-23, 1985) and guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Five mutants were treated every other day from the third to the sixth postnatal week with 10^6 units of human recombinant colony-stimulating factor-1 (CSF-1; Halenbeck *et al.*, 1989 kindly provided by Chiron Corp., Emeryville, CA). These animals were used to evaluate the effects of CSF-1 on stress fibers in osteoblasts *in vivo* (see below). Ten mutants were treated with 10^6 units of CSF-1 for the first four postnatal days before calvarial cells were isolated to examine the effects of CSF-1 on actin mRNA isoform sorting in osteoblasts *in vitro* (see below). The dose and duration of CSF-1 treatment reduced skeletal sclerosis comparable to that in our earlier work (Marks *et al.*, 1992, 1993).

Tissue preparation and evaluation of actin stress fibers in osteoblasts *in vivo*

These procedures are identical to those described earlier (Watanabe *et al.*, 1997). Briefly, six-week-old rats (two untreated normals, two untreated mutants and seven CSF-1-treated mutants) were anesthetized (ketamine/xylazine at 10 mg/kg im) and perfused with a buffered salt solution (36°C) supplemented with 0.2% Triton X-100 followed by 0.1% glutaraldehyde and 0.2% Triton and then 2.5% glutaraldehyde in the same buffer. This sequential perfusion using Triton preserves ultrastructure while reducing the electron density of the cytoplasm

(compare Fig. 3a and b) so that its filamentous components can be visualized using transmission electron microscopy (TEM). Osteoblasts in longitudinal sections of the proximal tibial metaphysis were examined by light microscopy after embedment in epoxy resin to quantitate the percentage of the bone surface occupied by these cells. Using the histomorphometric software package *Osteomeasure* (Osteometrics, Atlanta, GA) at least 5.0 μ m of randomly selected surface of metaphyseal bone, within 2.5 μ m of the proximal tibial growth plate, was traced. The extent of this surface occupied by osteoblasts was measured and expressed as a percentage of bone surface length. Osteoblasts on calvarial bone were examined by TEM after small blocks of bone were isolated, demineralized, embedded in epoxy resin, sectioned and stained as previous described (Watanabe *et al.*, 1997). Photographs taken from 20 randomly selected osteoblasts from each group of untreated animals (to compare with previous findings) and 80 osteoblasts from CSF-1-treated mutants were used to measure ratios of the lengths of stress fiber bundles to the length of the adjacent cell surface next to bone matrix and to confirm the ratios of the four osteoblast phenotypes we have previously described in the *tl* mutation (Watanabe *et al.*, 1997). The significance of the differences in the percent of bone surface occupied by osteoblasts and between relative stress fiber lengths in osteoblasts from these three groups of animals was determined using the non-parametric Mann-Whitney test (Zar, 1974). A probability of less than 0.05 was considered to be statistically significant.

Osteoblast isolation and evaluation of actin mRNA isoform sorting *in vitro*

Primary cultures of osteoblast-like cells from 4-day-old untreated normal, untreated mutant and CSF-1-treated mutants were established as previously described (Jackson *et al.*, 1994). Calvariae were removed aseptically, minced and the cells released during sequential digestions with 2mg/ml collagenase (Boehringer-Mannheim, Indianapolis (N) and 0.25% trypsin (G1BCO, Grand Island, NY) were pooled, plated and cultured in minimal essential medium (MEM, G1BCO) and 10% heat-inactivated fetal calf serum with changes of medium every 48 h. (Watanabe *et al.*, 1998). On day 5, cells on coverslips were fixed, washed and stored at 4°C in 70% ethanol. *In situ* hybridization was used to determine the patterns of intracellular distribution of β and γ mRNAs using isoform-specific probes and procedures previously described (Kislauskis *et al.*, 1993; Watanabe *et al.*, 1998). Briefly, a

mixture of five isoform-specific oligonucleotides were end-labeled with digoxigenin dUTP by deoxynucleotide terminal transferase (Boehringer-Mannheim Biochemicals) and hybridized separately for each probe set. The rat β -actin and γ -actin oligonucleotide probe sequences have been published by us (Watanabe et al., 1998). Controls included incubations without probe.

The intracellular distribution of β - and γ -actin mRNAs was determined blindly by two individuals in 100 randomly selected cells each from cultures from untreated rats and 900 cells each from CSF-1 treated rats as previously described (Watanabe et al., 1998) and classified into one of six patterns of intracellular distribution as previously described (Watanabe et al., 1998). The results were expressed as mean percentage and standard deviation for each pattern, isoform and cell source. Differences between groups were analyzed using the two-tailed Students' *t* test.

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