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ARTICLES

Aberrant Gene Expression in Cultured Mammalian Bone Cells Demonstrates an Osteoblast Defect in Osteopetrosis

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Abstract Osteopetrosis is a skeletal condition in which a generalized radioopacity of bone is caused by reduced resorption of bone by osteoclasts. However, it has recently been shown that during skeletal development in several osteopetrotic rat mutations specific aberrations occur in gene expression reflecting the activity of the bone forming cells, osteoblasts, and the development of tissue organization. To evaluate their pathogenetic significance, progressive osteoblast differentiation was studied in vitro. Primary cultures of normal osteoblasts undergo a sequential expression of cell growth and tissue-related genes associated with development of skeletal tissue. We report that osteoblast cultures can be established from one of these mutants, toothless; that these cells in vitro exhibit similar aberrations in gene expression during cell proliferation and extracellular matrix formation and mineralization observed in vivo; and that an accelerated maturation sequence by mutant osteoblasts mimics the characteristic skeletal sclerosis of this disease. These data are the first direct evidence for an intrinsic osteoblast defect in osteopetrosis and establish an in vitro model for the study of heritable skeletal disorders.

Key words: osteoclast, gene regulation, rat, skeleton, osteopontin, osteocalcin, mineralization

Osteopetrosis, a metabolic disease of bone inherited in humans, is characterized by a generalized skeletal sclerosis due to reduced bone resorption, variable morbidity and mortality, and lack of highly effective treatment [Marks, 1984; Osier and Marks, 1992]. Experimental studies on a series of osteopetrotic mutations in laboratory animals have shown that a common feature of each is a different interception in the development or function of osteoclasts, the cellular mediators of bone resorption [Marks, 1992]. One such mutation in the rat, toothless (*tl/tl*), has few osteoclasts and macrophages, elevated serum levels of 1,25 dihydroxyvitamin D and mutant bone exhibits abnormalities in two proteins, reduced levels of osteocalcin, and elevated levels of osteopontin gene expression [Cotton and Gaines, 1974; Lian and Marks, 1990; Marks, 1977; Marks et al., 1992; Shalhoub et al., 1993; Zerwekh et al., 1987]. Treatment of *tl/tl* rats with human recombinant colony-stimulating factor-1 (CSF-1), a cytokine produced by osteoblasts and other cells [Horowitz et al., 1989], improves the skeletal manifestations and increases osteoclast and macrophage populations but not to normal levels [Marks et al., 1992; 1993].

Based on evidence that osteoclast biology is regulated at least in part by the cells which form bone, we have recently analyzed osteoblast gene expression as reflected by mRNA levels during development of the skeleton in this mutation [Shalhoub et al., 1991]. These data showed characteristic reductions in osteoblast proliferation, expression of cell-growth-related genes, type I collagen and osteocalcin mRNA levels, together with elevations in fibronectin and alkaline phosphatase in *tl/tl* rats. On the basis of these developmental modifications in gene expression we postulated that the osteoclast abnormalities in this mutation could result from aberrations in mutant osteoblasts. This conclusion is consistent with the well-documented signalling mechanisms provided by osteoblasts which mediate osteoclast function [Abe et al., 1988; Glowacki and Lian, 1987; Malone et al., 1982; McSheehy and Chambers, 1986]. In the present report we show that osteoblasts can be isolated from these

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mutants and grown in culture, that these cells maintain most of the functional aberrations observed in vivo, and that mutant osteoblasts produce the pathognomonic feature of the disease, a sclerotic skeleton, by an accelerated mineralization of extracellular matrix.

MATERIALS AND METHODS

Animals were derived from our colonies bred to maintain the toothless (*tl*) mutation and maintained and used in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals,* prepared by the ILAR, National Research Council (DHHS Publication No. NIH 86-23, 1985) and the guidelines of the Animal Care Committee of the University of Massachusetts Medical School. Mutants and normal littermates were distinguished radiographically [Schneider et al., 1979] at birth.

Calvarial cells were isolated from four or more animals of each genotype before the fourth postnatal day by sequential digestion with collagenase and trypsin using modifications of a method previously published [Aronow et al., 1990; Owen et al., 1990]. Briefly calvariae from neonatal rats (0–4 days old) were isolated and subjected to consecutive digestions of 5, 20, and 40 min at 37°C with shaking in 2 mg/ml collagenase A (Boehringer-Mannheim, Indianapolis, IN) and 0.25% trypsin (Gibco, Grand Island, NY). The cells from the second and third digests were pooled and plated in minimal essential medium (MEM: Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS) in 100 mm or 24-well plates at a density of 5×10^5 or 14.2×10^3 cells/dish, respectively. Ascorbic acid (25 μg/ml) was added to the cultures on day 3. Media from day 6 were supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate, and 10% FCS.

Cultures, maintained for up to 4 weeks, were sampled during the three principal developmental periods (proliferation, extracellular matrix maturation, and mineralization) [Aronow et al., 1990; Owen et al., 1990] of the osteoblastic phenotype [Bellows et al., 1986; Gerstenfeld et al., 1987; Stein et al., 1990] and studied using morphological, biochemical, and molecular methods. Media samples were taken every 3 days for osteocalcin analysis and cell samples were taken every 2 days to measure cell proliferation (3H-thymidine radioautography) and alkaline phosphatase. Osteoblast cultures were harvested at 8, 14, 18, 21, and 28 days and were (1) fixed and stained for histochemical analyses (alkaline phosphatase and mineralization), (2) frozen at −20°C for biochemical analyses (collagen, calcium, alkaline phosphatase, and DNA), and (3) frozen in liquid nitrogen and stored at −70°C for mRNA analyses (histone H4, collagen, alkaline phosphatase, osteocalcin, osteopontin, and fibronectin). Cultures were analyzed by light microscopic radioautography, scanning electron microscopy, biochemical measurements of cell proliferation, alkaline phosphatase, osteocalcin, and type I collagen and mRNA levels of osteoblast genes as previously described by us [Owen et al., 1990; Shalhoub et al., 1991]. Morphometric analyses of bone nodule number and size were performed using the *Osteomeasure* software program (Ostometrics, Atlanta, GA).

RESULTS

Morphologically, osteoblasts derived from normal littermates (Fig. 1A,B) showed at 14 days by light microscopy the development of discrete cellular nodules which stained histochemically for alkaline phosphatase and were surrounded by proliferating cells with little or no mineralization of nodules. Mutant osteoblast cultures at this time also contained nodules (Fig. 1C,D), but they tended to be smaller with fewer proliferating cells which were located predominantly within each nodule near regions undergoing mineralization. The mean number of nodules did not differ in mutant and normal cultures, (3.55 ± 2.98 and 4.5 ± 3.37 per 5.4 mm^2, respectively) but the area of mutant nodules was significantly ($P \leq 0.05$) smaller ($0.015\ mm^2 \pm 0.004$ vs. $0.058\ mm^2 \pm 0.04$). By scanning electron microscopy (Fig. 2) mature primary cultures showed distinct differences in surface phenotypes. Normal osteoblast cultures consisted of elevated cell nodules (Fig. 2A) whereas cultures of mutant osteoblasts remained in flattened configurations on culture dishes (Fig. 2B).

Biochemical analyses of these cultures at 2, 3, and 4 weeks (Fig. 3) showed no significant changes in DNA content (cellularity) or total accumulated collagen (data not shown). Alkaline phosphatase, an early marker of mineralization, rose in normal osteoblast cultures between the second and third weeks and remained high through the fourth week. In mutant cultures levels of this enzyme were significantly higher than normal at weeks 2 and 3 and dropped precipitously at week 4, an event known to occur later in normal heavily mineralized cultures

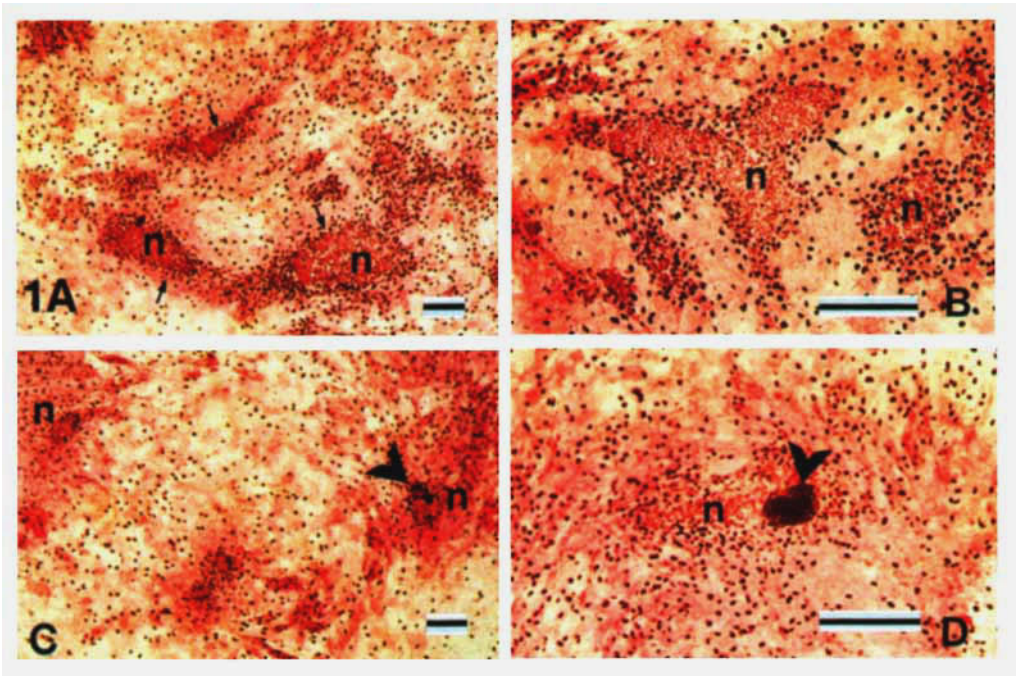

Fig. 1. Osteoblasts isolated from neonatal normal (**A, B**) and mutants (**C, D**) rats were cultured for 14 days on glass coverslips. Cultures were exposed to ^{3}H-thymidine (1 μCi/ml-Amersham, Arlington Heights, IL) the last 24 h, extensively washed, reacted for alkaline phosphatase (Sigma kit 86-R, Sigma Chemical Co., St. Louis, MO), dipped in liquid emulsion (Ilford K5), and radioautography performed under standard conditions. Alkaline phosphatase activity can be seen as a red or pink background and cells incorporating ^{3}H-thymidine as dark dots in each culture. In normal osteoblast cultures (A, B) discrete nodules (n) of osteoblasts are surrounded by proliferating cells (arrows). In mutant cultures nodules (n) are also present but mineralization (arrowhead) has already begun and proliferating cells tend to be located throughout nodules rather than at their periphery. Bars = 200 μm.

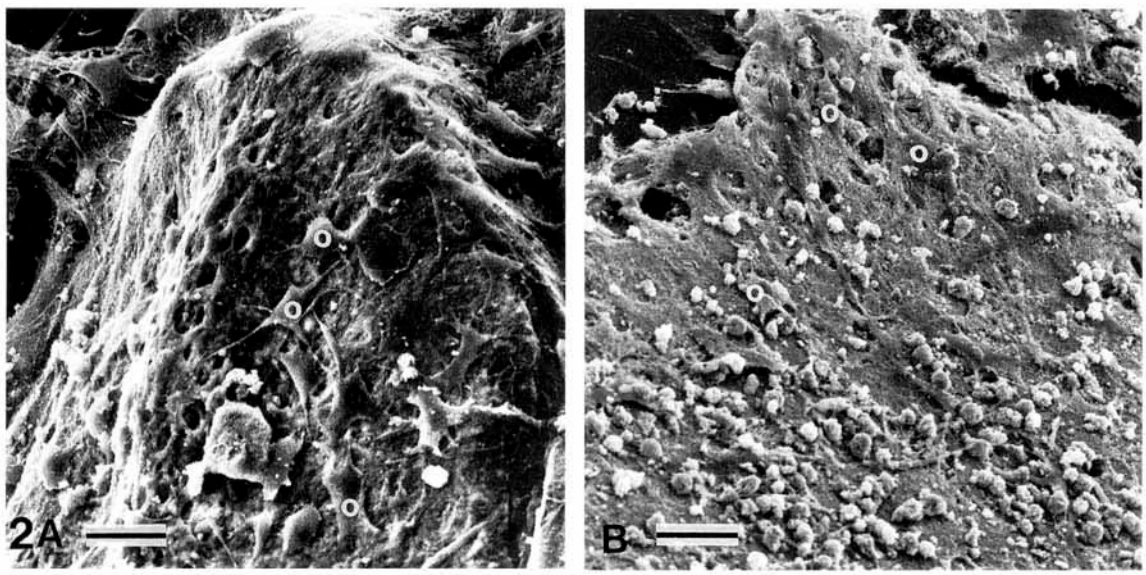

Fig. 2. Scanning electron micrographs of 30-day-old osteoblast (o) cultures from normal (**A**) and mutant (**B**) rats. Nodules seen in younger cultures (Fig. 1) continue to grow away from the surface of the culture dish in normal osteoblast cultures (A), producing mounds of cells in a mineralized matrix. Nodules in cultures of mutant osteoblasts (B) are much flatter than those in normal cultures (A). Bars = 10 μm.

[Aronow et al., 1990; Bellows et al., 1986; Gerstenfeld et al., 1987]. Osteocalcin, a later marker of mineralization, was low at 2 weeks and rose during the third week in both groups. During the fourth week production of this bone-specific protein increased significantly in normal osteoblast cultures but decreased in mutant cultures. The decrease in secreted osteocalcin in mutant cultures was not associated with an accumulation in the cell layer (data not shown). Early mineralization of mutant cultures is reflected at week 3 in mutant cultures by a 10-fold elevation of calcium, a major inorganic component of bone. This increased only slightly by week 4. Total calcium in normal cultures increased during the third week, but by the end of the culture period the calcium content of mutant cultures was still three-fold that in normal cultures.

We examined the extent to which the developmental expression of genes supporting osteoblast proliferation and differentiation parallel observed changes in cell growth and osteoblast-related proteins. Cellular mRNA levels were analyzed in normal and mutant cultures at 8, 18, and 28 days (Fig. 4). RNA from both genotypes was analyzed for each gene at the same time to permit direct comparison of normal and mutant values at all culture periods. Cell proliferation (histone H4 gene expression) is high in early stage primary cultures and undetectable by 28 days in both groups. Synthesis of Type 1 collagen, the principal protein of the extracellular matrix of bone, is elevated in mutants during the first week but reduced by the fourth week. Fibronectin mRNA levels fluctuate in both groups during culture but tend to be lower in mutants. Alkaline phosphatase mRNA levels are highest at 18 days in both cultures. At this time levels in normal cultures are greater than those in mutants. By 28 days mRNA levels have fallen in both groups but are higher in mutants. mRNA levels for osteopontin, a protein associated with osteoclast attachment, are maintained in normal cultures but are reduced in mutant cultures at 28 days. Osteocalcin levels are higher in mutants at day 8 and this persists to day 14 (data not shown). Osteocalcin peaks in both groups at 18 days but falls off faster in mutants thereafter.

A comparison of gene expression reflecting the developing bone cell phenotype in *tl/tl* osteoblasts in vivo and in vitro is shown in Figure 5, expressed as a percent of the values for normal littermates. In general, the changes in the time

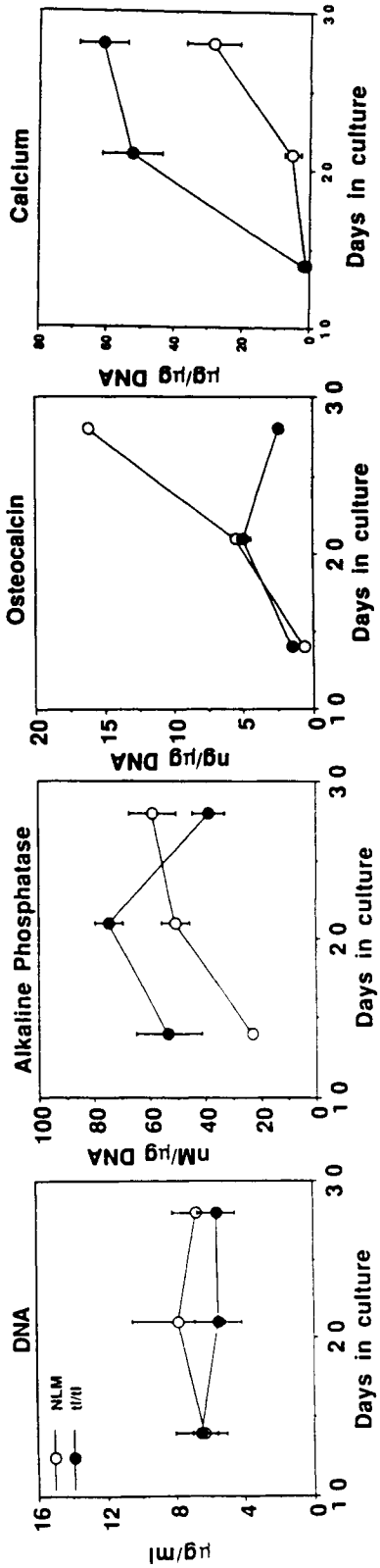

Fig. 3. Biochemical parameters of osteoblast cultures from normal (open circles) and mutant (solid circles) neonatal rats measured at 14, 21, and 28 days. After an earlier period of proliferation, DNA content of cultures is relatively unchanged in weeks 2–4 and not different in cultures of normal and mutant osteoblasts. Alkaline phosphatase is significantly elevated at weeks 2 and 3 in mutant cultures but falls during week 4. Osteocalcin increases significantly in normal cultures during week 4 when it is falling in mutant cultures. Calcium production increases in both groups during these culture periods.

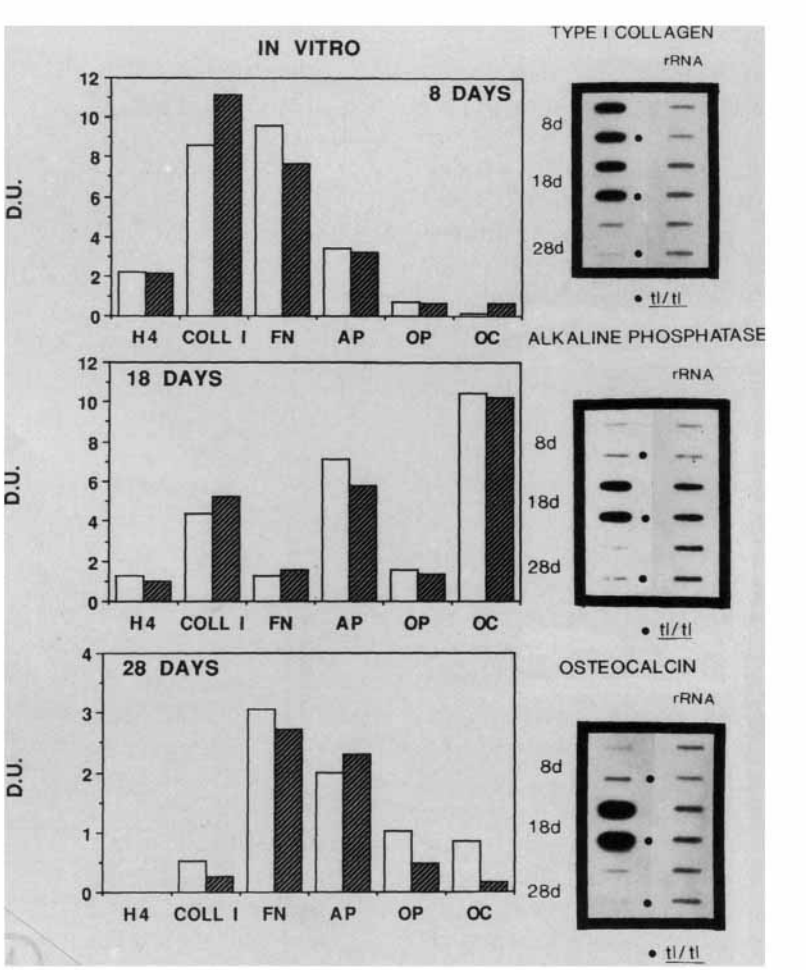

Fig. 4. Gene expression as reflected by mRNA levels in representative normal (open bars) and mutant (solid bars) osteoblast cultures at 8, 18, and 28 days. Total cellular RNA was isolated and mRNA levels determined in slot blots as previously described [Shalhoub et al., 1991] for histone H_4, collagen type I (coll 1), fibronectin (FN), alkaline phosphatase (AP), osteopontin (OP), and osteocalcin (OC), confirmed by Northern analyses and expressed as densitometric units (D.U.). Normal cultures exhibit early proliferation accompanied by high levels of gene expression for collagen and fibronectin. Later (18 days) alkaline phosphatase and osteocalcin are increased. Mutant cultures show earlier elevations (8 days) in collagen and osteocalcin and lower levels of collagen, osteopontin, and osteocalcin in 28-day cultures. Representative autoradiograms of selected genes are shown and include hybridization to a ribosomal RNA probe for normalization of data.

course and extent of gene expression as reflected in mRNA levels are similar in vivo and in vitro. Prominent among these are elevations in alkaline phosphatase, and depressions in type I collagen, osteopontin, and osteocalcin mRNA levels in mutants. Fibronectin levels are elevated in vivo and slightly reduced in vitro. Histone H4 levels are reduced in vivo and undetectable in both normal and mutant cultures in vitro. It should be noted that in 18 day cultures (Fig. 4) and in 14 day cultures by Northern blot analysis (data not shown), histone mRNA levels in mutant cultures are less than those in normal and at this time resemble the levels in vivo [Shalhoub et al., 1991].

DISCUSSION

The patterns of gene expression, cell proliferation, and extracellular matrix synthesis shown here for normal calvarial osteoblasts isolated from postnatal rats are identical to those shown for fetal rat osteoblasts isolated from the same skeletal site [Bellows et al., 1986; Aronow et al., 1990; Stein et al., 1990]. This includes sequential developmental periods characterized by cell proliferation, extracellular matrix production, maturation and mineralization, and the time course of synthesis of collagen, alkaline phosphatase, and osteocalcin. These same biochemical and morphological analyses indicate that mu-

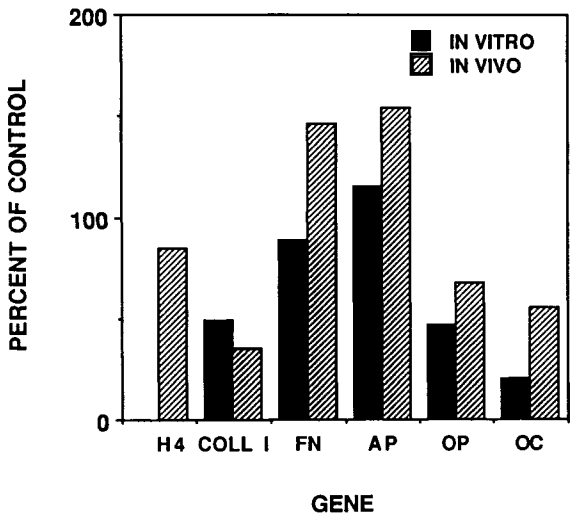

Fig. 5. Comparison of gene expression in mutant osteoblasts in vivo [Shalhoub et al., 1991] and in vitro at 28 days expressed as percent of that in normal littermates. Increases in alkaline phosphatase (AP) and decreases in type I collagen (coll I), osteopontin (OP), and osteocalcin (OC) in mutant osteoblasts in vivo are maintained in mutant osteoblast cultures. The discrepancies in histone H4 gene expression are related to the general synchrony of osteoblasts in vitro compared to a growing heterogenous population in vivo, and the fact that at the period examined in vitro osteoblast differentiation was proceeding with little or no cell proliferation (Fig. 4).

tant osteoblasts produce prematurely a heavily mineralized extracellular matrix which is the characteristic feature of the disease. In normal mature mineralized osteoblast cultures alkaline phosphatase and osteocalcin are down-regulated during weeks 5–7 [Aronow et al., 1990; Bellows et al., 1986; Gerstenfeld et al., 1987; Owen et al., 1990]. These events occur earlier (week 4) in mutant cultures. This precocious differentiation could result from the premature expression of factors which accelerate the expression of the osteoblast phenotype (i.e., mineralized nodules). Alternatively, the early, rapid rise in calcium deposition in mutant cultures may have induced signals which prevent further development of nodules, disrupting the normal temporal pattern of gene expression. Either way premature mineralization would produce the skeletal sclerosis characteristic of the disease. The effects of accelerated mineralization on the quantity and function of various bone matrix proteins, some of which are associated with osteoclast recruitment and activation, deserve further study.

These data taken together show that osteoblast cultures can be routinely established from calvariae of neonatal osteopetrotic (toothless) and normal rats, that these cultures exhibit reproducible patterns of cell proliferation, extracellular matrix production, and mineralization, and that the characteristic differences between mutant and normal osteoblast gene expression observed in vivo are preserved in cultured osteoblasts derived from each phenotype. The maintenance of these differences in a controlled culture environment, free of fluctuations in 1,25 dihydroxy vitamin D and other osteotrophic factors, is the first direct evidence for an intrinsic osteoblast abnormality in osteopetrosis and establishes this as an in vitro model for exploring mechanisms related to the aberrant osteoblast parameters of this particular type of metabolic bone disease. Because osteoblasts are known to secrete CSF-1 [Horowitz et al., 1989; Ohtsuki et al., 1992] and exogenous CSF-1 improves the skeletal manifestations of osteopetrosis in *tl/tl* rats [Marks et al., 1992, 1993], the characteristic aberrations in mutant osteoblast function may be related to reduced or inappropriate secretion of CSF-1. The ability to perpetuate this defect in culture will permit easier characterization of molecular parameters of the osteoblast aberrations and developmental responsiveness to physiological mediators in this mutation, as well as facilitate the development and testing of hypotheses for osteoblast-mediated regulation of bone resorption in vitro and in vivo.

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