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Original Article

Endochondral bone formation in toothless (osteopetrotic) rats: failures of chondrocyte patterning and type X collagen expression

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ABSTRACT The pacemaker of endochondral bone growth is cell division and hypertrophy of chondrocytes. The developmental stages of chondrocytes, characterized by the expression of collagen types II and X, are arranged in arrays across the growth zone. Mutations in *collagen II* and *X* genes as well as the absence of their gene products lead to different, altered patterns of chondrocyte stages which remain aligned across the growth plate (GP). Here we analyze GP of rats bearing the mutation toothless *(tl)* which, apart from bone defects, develop a progressive, severe chondrodystrophy during postnatal weeks 3 to 6. Mutant GP exhibited disorganized, non-aligned chondrocytes and mineralized metaphyseal bone but without cartilage mineralization or cartilaginous extensions into the metaphysis. Expression of mRNA coding for collagen types II (Col II) and X (Col X) was examined in the tibial GP by *in situ* hybridization. Mutant rats at 2 weeks exhibited Col II RNA expression and some hypertrophied chondrocytes (HC) but no Col X RNA was detected. By 3rd week, HC had largely disappeared from the central part of the mutant GP and Col II RNA expression was present but weak and in 2 separate bands. Peripherally the GP contained HC but without Col X RNA expression. This abnormal pattern was exacerbated by the fourth week. Bone mineralized but cartilage in the GP did not. These data suggest that the *tl* mutation involves a regulatory function for chondrocyte maturation, including Col X RNA synthesis and mineralization, and that the GP abnormalities are related to the Col X deficiency. The differences in patterning in the *tl* rat GP compared to direct *Col X* mutations may be explained by compensatory effects.

KEY WORDS: *collagen type II, collagen type X, chondrocyte, chondrodystrophy, osteopetrosis*

Introduction

The pacemaker of endochondral bone formation is cell division and hypertrophy of chondrocytes in growth plates (Farnum and Wilsman, 1989; Hunziker, 1994). It is the proliferation, vectorial extracellular matrix production and hypertrophy of chondrocytes together with mineralization of the longitudinally aligned matrix components that maintain the alignment and relative proportion of cell types during longitudinal bone growth (Vanky *et al.*, 1998). Furthermore, the mineralized cartilage columns derived from growth plates provide the scaffolding onto which metaphyseal bone matrix is secreted and mineralized by osteoblasts (Marks, 1998). In growth plate cartilages collagen type II (Col II) is synthesized by proliferating chondrocytes. When these cells differentiate into hypertrophied chondrocytes deeper in the growth plate, type II collagen synthesis ceases and type X collagen (Col X) begins to be produced (Iyama *et al.*, 1991; Schmid *et al.*, 1991). Thus, Col II and X are exclusively expressed in proliferating and hypertrophic chondrocytes, respectively (Castagnola *et al.*, 1986).

Osteopetrotic mutations are characterized by skeletal sclerosis resulting from interceptions of the development and/or function of bone-resorbing cells, osteoclasts (Popoff and Marks, 1995). In specific mutations a variety of other cells/tissues may be affected.

Abbreviations used in this paper: Col II, collagen type II; Col X, collagen type X; CSF-1, colony-stimulating factor-1; DIG, digoxygenin; GP, growth plate; HC, hypertrophied chondrocyte; RHT, ruthenium hexamine trichloride; tl, toothless mutation in the rat.

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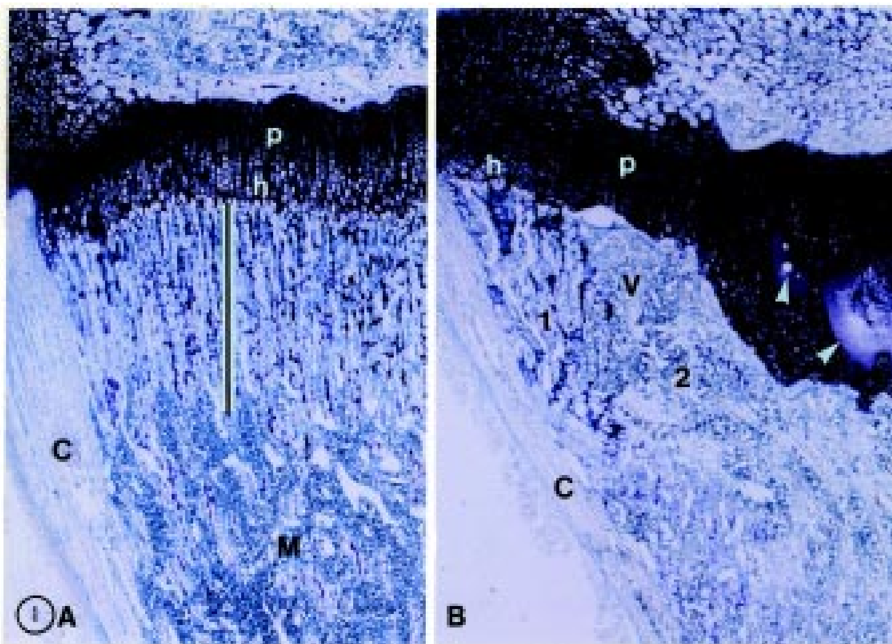


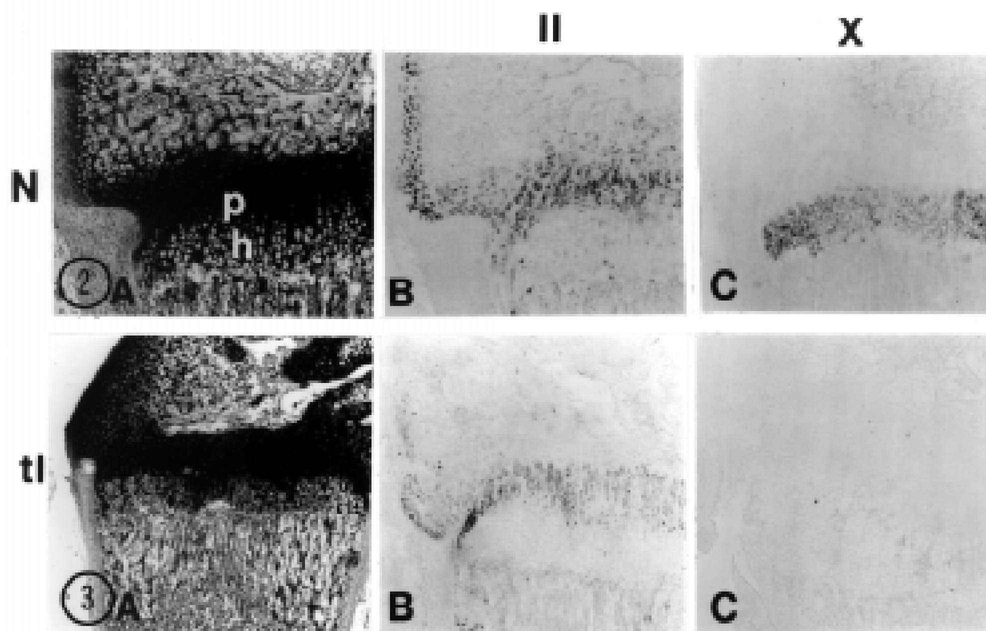
Fig. 1. Representative photomicrographs of the proximal tibia from 4-week-old normal (A) and toothless (B) rats differentially stained with toluidine blue. *The growth plate cartilage (purple) is toward the top of each figure and zones of proliferating (p) and hypertrophied (h) chondrocytes are indicated. In normal rats the growth plate cartilage is linear and of uniform thickness and cellular composition. The lateral cortical bone (c) is thick and the metaphyseal trabeculae (in area delineated by the vertical line) have cores of calcified cartilage (purple stain in this area). A marrow cavity (M) can be seen in the lower part of the figure (1A). In osteopetrotic (toothless-tl) mutants (1B) there are no marrow cavities, cortical bone (C) is thin and the growth plate cartilage is irregular in both shape and composition. Laterally, zones of proliferating (p) and hypertrophied (h) chondrocytes are present but centrally the growth plate is greatly thickened and includes areas of cell degeneration (arrowheads) and few hypertrophied chondrocytes. In the metaphysis, a lateral area (1) has cores of mineralized cartilage but the central region (2) has bone with few such cartilage remnants and irregular dilated venous (V) sinuses. Toluidine blue, X45.*

In the toothless *(tl)* mutation in the rat (Marks, 1977) a chondrodystrophy (Seifert 1994; 1996) develops by the third postnatal week. Injections of colony-stimulating factor-1 (CSF-1) improve the skeletal sclerosis (Marks *et al.,* 1993, 1992) but are without any effect on the chondrodystrophy (Odgren *et al.*, 1999). This chondrodystrophy in *tl* rats affects the development of cartilage and bone cells and endochondral ossification (Seifert, 1996). Specifically the zone of proliferating chondrocytes increases with age and the hypertrophic chondrocyte zone decreases, both effects beginning and being most obvious in the central regions.

Given these abnormalities in chondrocyte differentiation in growth plates of *tl* rats, we have compared postnatal gene expression for collagens type II and X, regional chondrocyte morphology and cartilage mineralization in tibial growth plates from mutant and normal rats. These data are expected to clarify the interdependent contributions of cartilage and bone cells during skeletal development.

Results

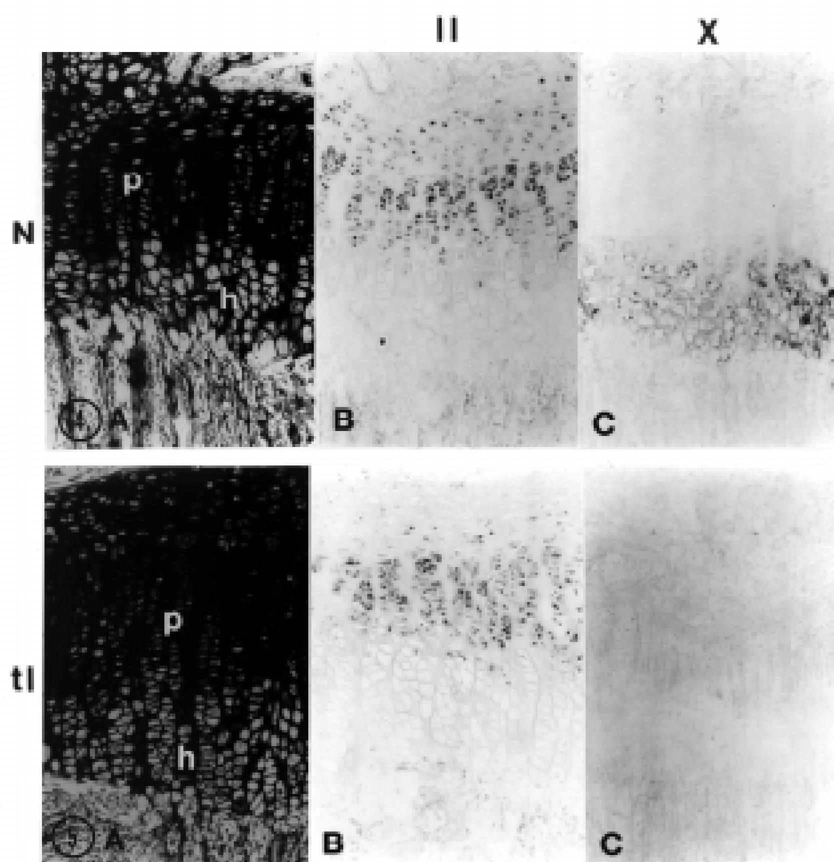
Figure 1 demonstrates the differences in organization and composition of the proximal tibial epiphyseal plates and metaphyses in normal rats and osteopetrotic littermates one month after birth. The growth plate in normal rats (Fig. 1A) is well organized throughout with linear progressions of proliferating and hypertrophied chondrocytes and a matrix that stains metachromatically



Figs. 2 and 3. Representative photomicrographs of the proximal tibia from a 2-week-old normal rat (N-Fig. 2) and its toothless (*tl*-Fig. 3) littermate stained with toluidine blue (A) and illustrating gene expression by *in situ* hybridization for collagens type II (B) and X (C) in adjacent sections. *Notice that there is no mRNA expression for collagen type X in tl rats (Fig. 3C). X40.*

dark blue in this figure. The metaphysis is of uniform thickness and its bone is formed on scaffolds of calcified cartilage, producing trabeculae with cores of metachromatic cartilage matrix. A well developed marrow cavity can be seen toward the center of the tibia and it and the metaphysis are supported laterally by a thick cortex of bone. In *tl* mutants (Fig. 1B) the growth plate is irregular in both composition and thickness. Proliferating chondrocytes can be seen throughout the growth plate but columns of hypertrophied cells are present only peripherally. Centrally the growth plate has local thickenings which include areas of cell degeneration without metachromatic cartilage matrix. The metaphysis reflects these manifestations in the growth plate. Laterally the bony trabeculae have cores of cartilage but centrally the typical metachromatic staining of cartilage matrix is absent. Instead, new bone forms here without a preexisting cartilage scaffold and vascular sinuses are prominent. There is no clearly delineated marrow cavity and cortical bone in mutants is thinner than in normal littermates.

Given the obvious distortions of form and composition in the growth plates and metaphyses in 4-week-old mutants, we evaluated chondrocyte function at earlier ages using probes for collagens II and X which are normally produced by growth plate chondrocytes in different phases of their differentiation. The expression of Col II and Col X in the proximal tibial growth plate of 2-week-old normal rats is shown in Figure 2. In these rapidly growing bones, Col II RNA is expressed by proliferating chondrocytes of the growth plate (Fig. 2A,B) and Col X RNA is expressed by the hypertrophied chondrocytes (Fig. 2A,C). In 2-week-old *tl* rats (Fig. 3) Col II RNA is expressed by proliferating chondrocytes (Fig. 3A,B) but less intensely than in normal rats. Col X RNA is not expressed by chondrocytes in the mutant skeleton (Fig. 3C), even though hypertrophied chondrocytes can be identified in the mutant growth plate (Fig. 3A).

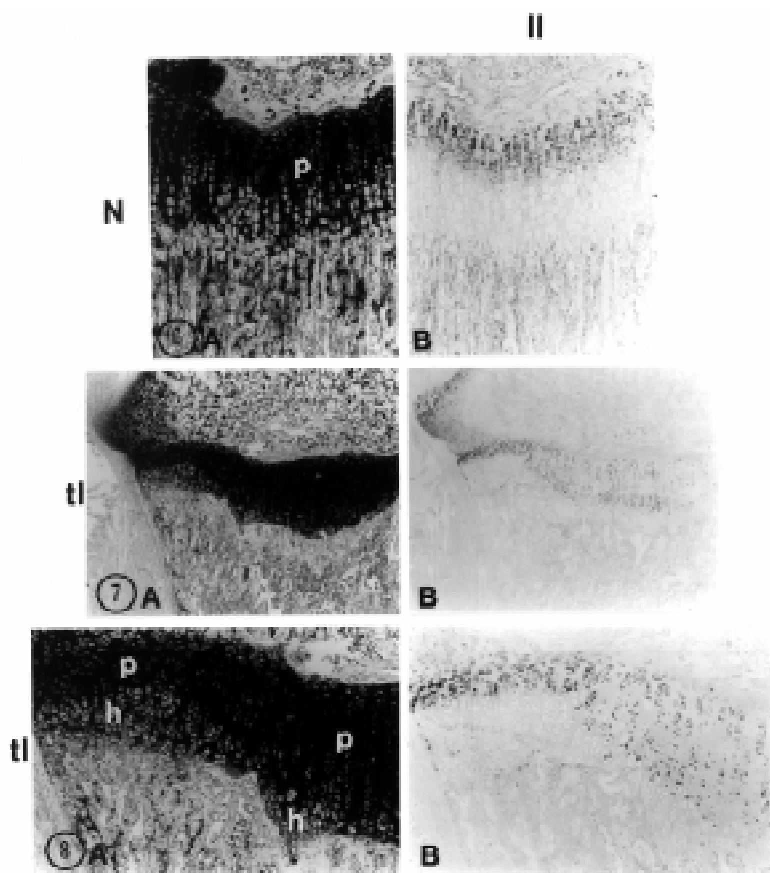


Figs. 4 and 5. Representative photomicrographs of the lateral part of proximal tibial growth plate from a 2-week-old normal rat (N-Fig. 4) and a toothless rat (*tl*-Fig. 5) at higher magnification than shown in the previous figures. *Sections are stained with toluidine blue (A) or incubated with probes to show by in situ hybridization expression of mRNA for collagens II (II-B) and X (X-C) in adjacent sections. Notice that in normal rats (Fig. 4) cells in the zone of proliferating (p) chondrocytes express type II collagen (B) and those in the zone of hypertrophy (h) express type X (C). In tl rats (Fig. 5), the expression pattern for type II collagen is normal but the hypertrophied (h) chondrocytes do not express type X collagen. X95.*

The differential expression of Col II and X in normal and mutant growth plates is shown at higher magnification in Figures 4 and 5, respectively. Here one can see that in normal rats Col II RNA is highly expressed by a band of young proliferating chondrocytes (Fig. 4A,B). Nearer to the zone of hypertrophy expression for this gene is reduced, and it is not detected in hypertrophied chondrocytes (Fig. 4B). In contrast, Col X RNA in normal rats is expressed exclusively by hypertrophied chondrocytes (Fig. 4A,C), and this expression ends abruptly at the chondro-osseous junction where the growth plate abuts the proximal tibial metaphysis. In 2-week-old *tl* rats (Fig. 5) Col II is expressed by proliferating chondrocytes (Fig. 5A,B) but lacks the intense band of expression seen in the young proliferating cells in normal littermates (Fig. 4B). There is no expression of Col X RNA by mutant chondrocytes (Fig. 4C) even though this section contains hypertrophied chondrocytes (Fig. 4A).

Next we evaluated gene expression for these two collagens in the proximal tibial growth plates of 3-week-old animals. Expression of Col X RNA was not detectable in mutant growth plates (data not shown). Thus, the results at this age for Col X were identical to what we found in 2-week-old animals (Figs. 4C and 5C). Col II RNA expression in normal 3-week-old rats (Fig. 6) was compact, confined to the zone of proliferating chondrocytes (Fig. 6A,B) and was most intense in the younger proliferating cell population. In mutants (Figs. 7 and 8), Col II expression differed in the lateral and central regions of the proximal tibial growth plate. In the lateral growth plate Col II expression was linear and confined to proliferating cells (Fig. 7A,B). Centrally, Col II expression spread throughout the growth plate and was much less intense than its expression laterally in mutants. At higher magnification (Fig. 8) one can clearly see the lack of uniform expression of Col II in the central growth plate (Fig. 8B) where two bands of higher expression are separated by a region of low expression (rt. side of Fig. 8B). Thus, instead of the suppressed expression of Col II during the differentiation of proliferating normal chondrocytes (Fig. 6B), mutant chondrocytes exhibit cyclical expression for Col II in a thickened growth plate with few hypertrophic cells (right sides of Fig. 8A and B).

Based on the reported relationship between Col X and mineralization in cartilage (Iyama *et al.,* 1991; Kirsch and Von der Mark, 1991; Schmid *et al.,*1991), we examined the extent of mineraliza-



Figs. 6,7 and 8. Representative photomicrographs of the proximal tibia from a 3-week-old normal rat (N-Fig. 6) and a toothless littermate (*tl*-Figs. 7 and 8). *Sections in panel A are stained with toluidine blue and those in panel B show the expression of type II (11) collagen mRNA in adjacent sections by in situ hybridization. In normal rats (Fig. 6) collagen II expression (B) is linear and confined to the zone of proliferating (p) chondrocytes. In toothless rats, the growth plate remains irregular in thickness with hypertrophied chondrocytes (h) concentrated at the periphery and absent from most central areas (Fig. 7A). Gene expression for type II collagen (Figs. 7B and 8B) is restricted to proliferating (p) chondrocytes laterally but expressed by some cells in early hypertrophy centrally (Fig. 8B). X85 in Figures 6 and 8; X 40 in Figure 7.*

tion in growth plates of mutants (Fig. 9). Mutant cartilage exhibited no mineralization and regions of cell degeneration in central areas (Fig. 9A), where wide venous sinuses pressed directly onto the unmineralized matrix. However, adjacent metaphyseal bone was mineralized (Fig. 9B) in the mutant skeleton. These data demonstrate a selective deficit of cartilage mineralization in the *tl* skeleton. The regions of cell degeneration seen in the more mature (lower) tibial growth plate of mutants (Figs. 1B and 9A) are preceded by pyknotic cell clusters in younger parts of the growth plate (Fig. 10). These degenerating cell groups appear early in the zone of proliferation, suggesting that progeny of some cell divisions are contracted and aborted soon after cell division is initiated in the growth plate. Remnants of these degenerating cell clusters can be seen more distally in the growth plate, together with intercellular areas that stain weakly. Hypertrophied chondrocytes are small in both number and size in the central regions of the mutant growth plate (Fig. 10). In the normal growth plate cell death is only seen in the terminal hypertrophied chondrocytes at the chondro-osseous junction (Farnum and Wilsman, 1989; Hunziker, 1994).

In summary, these data demonstrate that in *tl* rats: 1) type X collagen RNA is not expressed in the growth plate, even though hypertrophic chondrocytes are present, albeit in reduced number and uneven distribution, 2) type II collagen expression in growth plates is present but irregular; compact in the lateral part of the growth plate and cyclical and diffuse centrally, 3) cartilage mineralization fails but bone does mineralize, 4) centrally, cartilage does not extend into the metaphysis (presumably because of the failure of chondrocyte hypertrophy) and bone trabeculae form here without a cartilage scaffold, 5) local areas of pyknotic chondrocyte clusters early in the proliferative zone are followed by regions of degenerating cells and restricted chondrocyte hypertrophy deeper in the growth plate.

Discussion

During long bone growth endochondral ossification proceeds through consecutive steps of concurrent chondrocyte proliferation and production of Col II followed by down-regulation of Col II, hypertrophy and production of Col X. Recent data indicate that 80% of the growth in murine bones is related to chondrocyte hypertrophy (Vanky *et al.,* 1998). Thus, it is not surprising that in *tl* rats where few chondrocytes hypertrophy, long bone growth is retarded (Seifert, 1996; Odgren *et al.,* 1999). Mineralization of cartilage normally begins in the early hypertrophic zone and is mediated by matrix vesicles from these cells. The regulation of chondrocyte maturation has recently been shown to result from the local interactions of Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) and its receptor (Kronenberg *et al.,* 1997). The expression of Ihh in maturing chondrocytes upregulates PTHrP secretion from the perichondrium which, in turn, acts as a paracrine suppressor of chondrocyte hypertrophy through PTHrP receptors on prehypertrophic cells (Lee *et al.,* 1996; Philbrick *et al.,* 1996; Vortkamp *et al.,* 1996, 1998; Zou *et al.,* 1997; Philbrick 1998). Hypertrophic chondrocytes also slow the maturation of their own precursors in a negative-feedback loop and delay apoptosis by upregulating Bcl-2 expression (Amling *et al.,* 1997). Recently, BMP-6 has been shown to be a positive regulator of avian chondrocyte differentiation (Grimsrud *et al.,* 1999). These mechanisms produce a growth plate with uniformly thick zones of chondrocyte proliferation and hypertrophy (Fig. 1A). In the absence of PTHrP, growth plates are thin and mineralization accelerated (Amizuka *et al.,* 1994; Karaplis *et al.,* 1994; Weir *et al.,* 1996). Maturation and death of hypertrophic chondrocytes occurs, at least in part, by signals from endothelial cells at the chondro-osseous junction (Bittner *et al.,* 1998) and involves the secretion of enzymes that cross-link a variety of matrix molecules (Nurminskaya *et al.,* 1998), stabilizing the mineralized cartilaginous scaffold onto which osteoblasts deposit metaphyseal bone. In *tl* rats, proliferation of some central chondrocytes is aborted, differentiation and hypertrophy of the remainder are delayed, expression of Col II is irregularly distributed and extended, and expression of Col X never occurs. Mineralization of cartilage fails, there is no cartilaginous scaffolding for bone formation in the central metaphysis and bone here forms without a cartilaginous core (Fig. 1B). Laterally, the growth plate

distortions are not as spectacular presumably because of adjacent periosteal collar influences on chondrocyte differentiation (Bianco *et al.,* 1998; Long and Linsenmayer, 1998). Nevertheless, Col X expression also fails in this region of the mutant growth plate. It has recently been shown (Zerega *et al.,* 1999) that the addition of PTHrP to cultures of hypertrophied avian chondrocytes suppresses both expression of Col X and mineralization by these cells. Given the perichondrial location of PTHrP secreting cells in growing long bones (Zou *et al.,* 1997), the failure of Col X expression and mineralization of cartilage by hypertrophied chondrocytes in the lateral areas of the growth plate of *tl* rats could be due to overexpression of PTHrP by adjacent perichondrial cells. Explaining the pathology of central areas of the *tl* growth plate (Fig. 1B) is more difficult and may involve abnormalities in the PTHrP-Ihh axis, BMP-6 and its receptor or Bcl-2 expression. Thus, expression of these genes needs to be evaluated systematically in the *tl* growth plate during development to understand the mechanisms for regional differences in chondrocyte maturation. Furthermore, a comprehensive analysis of the mechanisms of the premature cell death and loss of chondrocytes in the mutant growth plate is warranted.

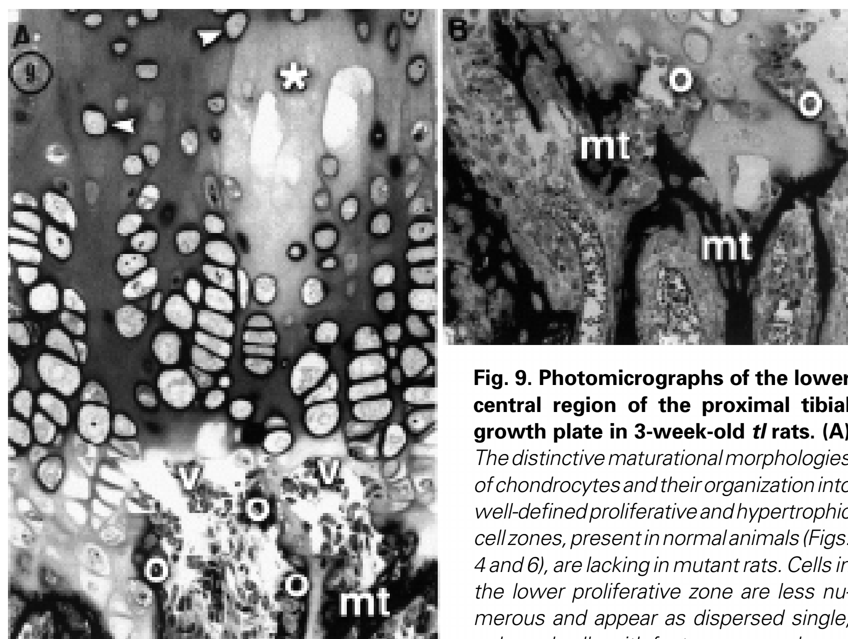


Fig. 9. Photomicrographs of the lower central region of the proximal tibial growth plate in 3-week-old *tl* rats. (A) *The distinctive maturational morphologies of chondrocytes and their organization into well-defined proliferative and hypertrophic cell zones, present in normal animals (Figs. 4 and 6), are lacking in mutant rats. Cells in the lower proliferative zone are less numerous and appear as dispersed single, enlarged cells with features more characteristic of hypertrophic chondrocytes (arrowheads). Areas of necrosis, acellularity and loss of metachromasia (asterisk) indicate focal, interstitial loss of cartilage cells and matrix. This is presumably due to premature chondrocyte death which releases enzymes that enlarge lacunae and degrade the proteoglycans responsible for the metachromatic staining of these areas with toluidine blue. The cells representing the hypertrophic cell zone show a mixed expression of morphologies and here cartilage matrix fails to mineralize (no black deposits of silver ions from the Von Kossa reaction, which shows that bone (mt) does mineralize at the bottom of this figure). Large, dilated metaphyseal vessels (v) irregularly invade this region. Numerous osteoblasts (o) directly line unmineralized cartilage trabeculae or the metaphyseal surface of the growth plate.* **(B)** *Osteoblasts deposit an osteoid matrix on the metaphyseal surface of the growth plate or along unmineralized cartilage trabeculae which mineralizes to form irregularly shaped trabeculae (mt). Toluidine blue/von Kossa. X125.*

Type X collagen is a short, non-fibrillar collagen synthesized by hypertrophic chondrocytes before mineralization and vascular invasion of the growth plate (Iyama *et al.,* 1991; Kirsch and Von der Mark, 1991; Reichenberger *et al.,* 1991; Schmid *et al.,* 1991; Claassen and Kirsch, 1994). Its appearance only after chondrocyte hypertrophy, together with its expression by chondrocytes in sites of mineralization at tendon insertions (Niyibizi *et al.,* 1996) and osteoarthritic transformation of articular cartilage (Hoyland *et al.,* 1991), have suggested a role for type X collagen in mineralization. Type X collagen is found in two forms in the extracellular matrix (Schmid and Linsenmayer, 1990): as fine filaments adjacent to hypertrophic chondrocytes and in the interstitial matrix in association with type II collagen (Chen *et al.,* 1992). Degradation of Col X occurs in the metaphysis by the combined actions of degradative enzymes present in osteoclasts (Sires *et al.,* 1995; Vu *et al.,* 1998).

Mutations in the *Col X* gene lead to metaphyseal chondroplasias characterized by thin growth plates and metaphyses (Jacenko *et al.,* 1993; Olsen, 1995; Pokharel *et al.,* 1995) quite opposite the skeletal manifestations in *tl* rats (Seifert, 1994, 1996). Cloning of the mouse gene (Apte *et al.,* 1992; Elima *et al.,* 1993) made Col X-deficient mice available for analysis. Early studies revealed (Rosati *et al.,* 1994) no gross abnormalities in bone growth or development in these mice, but subsequent analyses (Kwan *et al.,* 1997) showed reductions in resting chondrocytes and articular cartilage thickness and a shift of matrix vesicles and proteoglycans from the zone of hypertrophy to earlier stages in chondrocyte differentiation. Furthermore, *Col X* null mice show no abnormalities in chondrocyte morphology or differentiation patterns including premature apoptosis (Kwan *et al.,* 1997). These subtle deficiencies in Col X deficient mice contrast with the manifestations in *tl* rats where failure to express Col X is associated with a failure of mineralization and irregular, thick growth plates.

The chondrodystrophy that is characteristic of the *tl* mutation (Seifert, 1994, 1996) appears to result from central regional derangements in chondrocyte patterning (proliferation and hypertrophy) and gene expression. Hypertrophic chondrocytes occur in reduced numbers and in skewed distributions in tibial growth plates, and fail to express Col X RNA. Col II RNA is present, not in well-aligned layers of cells, but rather concentrated to lateral parts of the tibia. These derangements could be caused by deficiencies in the cartilage matrix, which affect cartilage mineralization and intercept recognition or adhesion by different cells. Osteoblasts do not populate cartilage scaffolds in the metaphysis of *tl* rats, and bone trabeculae are formed without a cartilage scaffold. Also in cartilage itself, local clusters of pyknotic chondrocytes appear early in the proliferative zone, followed by regions of cell degeneration and restricted hypertrophy toward the metaphysis. Both phenomena may be mediated by the failure of vascularization, i.e. the interaction of endothelial cells with cartilage matrix. This failure could interfere with both the supply of nutrients and oxygen to chondrocytes and with the entrance of osteoblast precursors. The end result of these abnormalities in chondrocyte development is the retardation of long bone growth, presumably mediated by the failure of the growth plate to provide a mineralized cartilage surface for the orderly attraction and differentiation of bone cell precursors

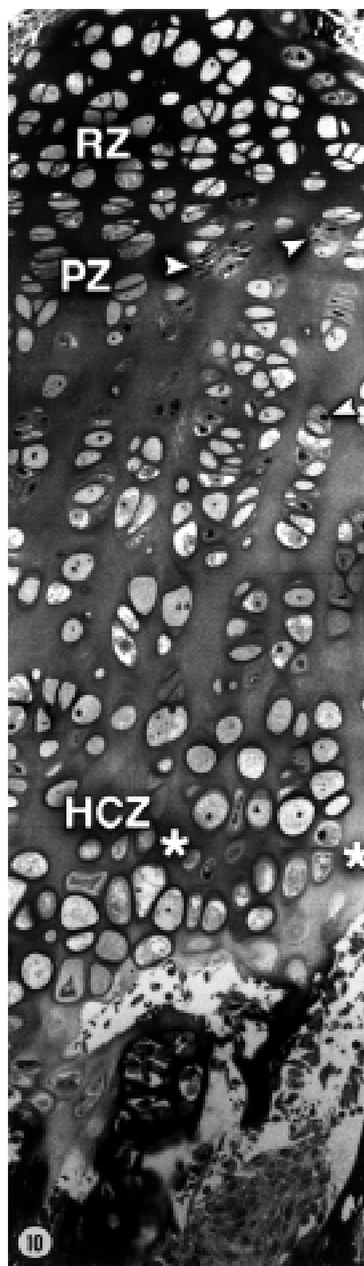


Fig. 10. A montage of the entire thickness of the central region of tibial growth plate from a 3-week-old *tl* rat showing the resting (RZ), proliferative (PZ) and hypertrophic (HCZ) cell zones. *Several cells within isogenous cell clusters located in the upper proliferative zone show evidence of cell death (pycnotic nuclei, vacuolated cytoplasm) (arrowheads). This premature cell death may cause the reduction in cartilage cellularity and the abnormal zonal organization. Cell orientation is disrupted in the HCZ and longitudinal septae here fail to mineralize (asterisk). The lack of organization of these cells into orderly columns possessing transverse septae may interfere with normal vascular invasion. Toluidine blue/von Kossa. X135.*

(Marks, 1997). The cortices of all growing bones are initially thin and they thicken during remodeling of the primary bone (Marks, 1998). This remodeling is blocked in the *tl* rat, due to depressed bone resorption. Thus, thin cortices and growth retardation coexist in this mutation due to abnormalities in bone resorption and chondrocyte differentiation, respectively.

In summary, we have shown that in the *tl* mutation in the rat hypertrophic chondrocytes are few in number, fail to express Col X and cartilage does not mineralize. Furthermore, premature degeneration of some early proliferating chondrocyte clusters produces a distorted growth plate in whose central regions chondrocytes continue to irregularly produce Col II mRNA but not cartilage cores for metaphyseal bone. The chondrometaphyseal dysplasia that results compromises skeletal growth and disrupts the currently unspecified interdependent messages from both cartilage and metaphyseal bone that produce the predictable, coordinated differentiation of cartilage and bone cells that mediate longitudinal bone growth. Given the abnormalities in cartilage (Seifert, 1994, 1996) and bone (Seifert *et al.,* 1988; Shalhoub *et al.,* 1991; Sundquist *et al.,* 1995; Wisner-Lynch *et al.,* 1995; Marks, 1997; Watanabe *et al.,* 1997, 1998) cells and in the skeletal vasculature (Aharinejad *et al.,* 1995), this mutation will be a valuable model for exploring the roles of these cells and tissues in the development and maintenance of the skeleton. How a single, autosomal recessive mutation in *tl* rats produces such a unique constellation of effects including compensatory adjustments remains to be established.

Materials and Methods

All the experiments performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical Center (UMMC). Animals were maintained and used at the UMMC according to the recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86-23, 1985).

Source of animals and tissue processing

Rats were obtained from our breeding colonies at the UMMC and mutants *(tl/tl)* were identified radiographically at birth (Schneider *et al.,* 1979) by the failure of development of marrow cavities in long bones. Normal homozygotes (+/+) and heterozygotes (*tl*/+) are distinguishable only by breeding, and normal littermates included both these genotypes. Rats of normal and mutant phenotypes were killed by cervical dislocation at 2,3 and 4 weeks after birth. The proximal tibiae were quickly isolated, cleaned of muscles, bisected in the sagittal plane and immersion fixed in freshly prepared 4% paraformaldehyde in RNase-free phosphate-buffered saline, pH 7.0, at 4ºC for 24 h. Bisected tibiae including the proximal growth plate were demineralized in 0.3M EDTA, pH7.0, at 4ºC, dehydrated and vacuum-embedded in paraffin. Serial sections were cut at 7 μm, floated on RNase-free water and mounted on clean glass slides pretreated with aminopropyl triethoxysilane (Sigma, St. Louis, MO). Blocks and glass-mounted sections were stored at 4ºC until used.

Tibiae were also removed and sectioned in the mid-sagittal plane. Under magnification, parallel 1-mm thick parasagittal slices containing the proximal growth plate cartilage and metaphysis were cut, fixed and processed undecalcified according to the method of Hunziker and Hermann (1982) (Hunziker, 1994) which optimally preserves chondrocytes in their fully expanded state. Briefly, slices were fixed for 2 h in 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, containing 0.7% ruthenium hexamine trichloride (RHT; Polysciences, Inc., Warrington, PA, USA). Slices were then rinsed in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.7% RHT. The presence of RHT, a low molecular weight cationic dye of high positive charge density, in the fixatives causes precipitation of pericellular proteoglycans and maintains plasma membrane bonds with the surrounding extralacunar matrix. This minimizes extraction of proteoglycans and the artifactual shrinkage of chondrocytes that accompany conventional aqueous fixation methods. Slices were dehydrated in ethanol and embedded in epoxy resin. Semi-thin sections (1 μm) were cut and stained for mineral by the von Kossa method (Seifert, 1996) and counterstained in 0.5% toluidine blue in 1% sodium borate.

Probes for hybridization

Clones containing rat Col II and X mRNA sequences were selected from a femur cDNA library as described, and inserts were sequenced (Wurtz *et al.,* 1998). Aligned sequences were deposited under the accession numbers AJ224879 and AJ131848, respectively, in the EMBL database.

RNA probes were synthesized in the presence of digoxygenin (DIG)-modified UTP by an *in vitro* transcription kit (Boehringer Mannheim, Mannheim, Germany) as directed by the manufacturer. Controls including

sense probes and the insert of pSPT19-Neo (Boehringer Mannheim) were also transcribed. The oligonucleotides transcribed for Collagen αI (II) and Collagen αI (X) were specific and showed no crossreactivity (Wurtz *et al.,* 1998; and unpublished data).

***In situ* hybridization**

The sections obtained from sagitally sectioned tibiae were reacted with the DIG-labeled riboprobes for Col II, Col X or control probes in an *in situ* hybridization protocol developed at the Center for Oral Biology specifically for analyzing gene expression in mineralized tissues. This method has been described (Liao *et al.,* 1998; Wurtz *et al.,* 1998; Marks *et al.,* 1999). Briefly, deparaffinized sections were exposed to 0.2M HCl, proteinase K and glycine, post fixed in 4% paraformaldehyde, reacted with triethanolamine and hybridized overnight at 42ºC in a sealed humid chamber while exposed to 0.5 ng DIG-UTP-labeled riboprobe/μL. High stringency washes and RNase treatment preceded identification of specifically bound DIG-labeled riboprobes by reacting with an alkaline phosphatase-coupled DIG antibody (Boehringer Mannheim). Exposure times for development ranged from 100 to 180 min depending upon the probe and the age of the animal.

Gene expression for *Col II* and *X* in the tibial growth plate was evaluated in adjacent sections of the same block for each probe and every fifth section was stained with toluidine blue to identify more precisely the cell populations expressing each gene. Sections from normal and mutant rats of the same age were examined by *in situ* hybridization at the same time, facilitating comparisons. At least 5 sections for each probe from at least three animals of each age and genotype were analyzed to determine the sites, times, intensity and patterns of gene expression for *Col II* and *X* in the proximal epiphyseal plate. All control probes were negative.

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References

AHARINEJAD, S., MARKS, S.C. Jr., BICK, P., MASON-SAVAS, A., MACKAY, C.A., LARSON, E.K., JACKSON, M.E., LUFTENSTEINER, M. and WIESBAUER, E. (1995). CSF-1 treatment promotes angiogenesis in the metaphysis of osteopetrotic (toothless, *tl*) rats. *Bone 16*: 315-324.

AMIZUKA, N., WASHAWSKY, H., HENDERSON, J.E., GOLTZMAN, D. and KARAPLIS, A.C. (1994). Parathyroid hormone related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J. Cell Biol. 126*: 1611-1623.

AMLING, M., NEFF, L., TANAKA, S., INOUE, D., KUIDA, K., WEIR, E., PHILBRICK, W.M., BROADUS, A.E. and BARON, R. (1997). Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development. *J. Cell Biol. 136*: 205-213.

APTE, S.S., SELDIN, M.F., HAYASHI, M. and OLSEN, B.R. (1992). Cloning of the human and mouse type X collagen genes and mapping of the mouse type X collagen gene to chromosome 10. *Eur. J. Biochem. 206*: 217-224.

BIANCO, P., CANCEDDA, F.D., RIMINUCCI, M. and CANDEDDA, R. (1998). Bone formation via cartilage models: the “borderline” chondrocyte. *Matrix Biol. 17*: 185-192.

BITTNER, K., VISCHER, P., BARTHOMES, P. and BRUCKNER, P. (1998). Role of the subchondral vascular system in endochondral ossification: endothelial cells specifically derepress late differentiation in resting chondrocytes *in vitro. Exp. Cell Res. 238*: 491-497.

CASTAGNOLA, P., MORO, G., DESCALZI-CANCEDDA, F. and CANCEDDA, R. (1986). Type X collagen synthesis during *in vitro* development of chick embryo tibial chondrocytes. *J. Cell Biol. 102*: 2310-2317.

CLAASSEN, H. and KIRSCH, T. (1994). Immunolocalization of type X collagen before and after mineralization of human thyroid cartilage. *Histochemistry 101*: 27-32.

CHEN, Q., FITCH, J., LINSENMAYER, C. and LINSENMAYER, T.F. (1992). Type X collagen: covalent crosslinking to hypertrophic cartilage-collagen fibrils. *Bone Miner. 17*: 223-227.

ELIMA, K., EEROLA, I., ROSATI, R., METSÄRANTA, M., GAROFALO, S., PERÄLÄ, M., DE CROMBRUGGHE, B. and VUORIO, E. (1993). The mouse collagen X gene: complete nucleotide sequence, exon structure and expression pattern. *Biochem. J. 289*: 247-253.

FARNUM, C.E. and WILSMAN, N.J. (1989). Cellular turnover at the chondro-osseous junction of growth plate cartilage: analysis by serial sections at the light microscopical level. *J. Orthop. Res. 7*: 654-666.

GRIMSRUD, C.D., ROMANO, P.R., D'SOUZA, M., PUZAS, J.E., REYNOLDS, P.R., ROSIER, R.N. and O'KEEFE, R.J. (1999). BMP-6 is an autocrine stimulator of chondrocyte differentiation. *Bone Miner. Res. 14*: 475-482.

HOYLAND, J.A., THOMAS, T.J., DONN, R., MARRIOTT, A., AYAD, S., BOOT-HANFORD, R.P., GRANT, M.E. and FREEMONT, A.J. (1991) Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. *Bone Miner. 15*: 151-164.

HUNZIKER, E.B. (1994). Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. *Microsc. Res. Tech. 28*: 505-519.

HUNZIKER, E.B. and HERMANN, W. (1982). Improved cartilage fixation by ruthenium hexamine trichloride (RHT). *J. Ultrastruct. Res. 81*: 1-12.

IYAMA, K-I., NINOMIYA, Y., OLSEN, B.R., LINSENMAYER, T.F., TRELSTAD, R.L. and HAYASHI, M. (1991). Spatiotemporal pattern of type X collagen gene expression and collagen deposition in embryonic chick vertebrae undergoing endochondral ossification. *Anat. Rec. 229*: 462-472.

JACENKO, O., LUVALLE, P.A. and OLSEN, B.R. (1993). Spondylometaphyseal dysplasia in mice carrying a dominant negative mutation in a matrix protein specific for cartilage-to-bone transition. *Nature 365*: 56-61.

KARAPLIS, A.C., LUZ, A., GLOWACKI, J., BRONSON, R.T., TYBULEWICZ, V.L, KRONENBERG, H.M. and MULLIGAN, R,C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes. Dev. 8*: 277-289.

KIRSCH, T. and VON DER MARK, K. (1991) Isolation of human type X collagen and immunolocalization in fetal human cartilage. *Eur. J. Biochem. 196*: 575-580.

KRONENBERG, H.M., LEE, K., LANSKE, B. and SEGRE, G.V. (1997) Parathyroid hormone-related protein and Indian hedgehog control the pace of cartilage differentiation. *J. Endocrinol. 154*: S39-S45.

KWAN, K.M., PANG, M.K.M., ZHOU, S., COWAN, S.K., KONG, R.Y.C., PFORDTE, T, OLSEN, B.R., SILLENCE, D.O., TAM, P.P.L. and CHEAH, K.S.C. (1997) Abnormal compartmentalization of cartilage matrix components in mice lacking collagen X: implications for function. *J. Cell Biol. 136*: 459-471.

LEE, K., LANSKE, B., KARAPLIS, A.C., DEEDS, J.D., KOHNO, H., NISSENSON, H., KRONENBERG, R.A. and SEGRE, G.V. (1996). Parathyroid hormone-related peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology 137*: 5109-5118.

LIAO, H., BRANDSTEN, C., LUNDMARK, C., CHRISTERSSON, C. and WURTZ, T. (1998). Osteonectin RNA and collagen α 1 (I) RNA in the developing rat maxilla. *Eur. J. Oral. Sci. 106*: 418-423.

LONG, F. and LINSENMAYER, T.F. (1998) Regulation of growth region cartilage proliferation and differentiation by perichondrium. *Development 125*: 1067-1073.

MARKS, S.C. Jr. (1977). Osteopetrosis in the toothless *(tl)* rat: presence of osteoclasts but failure to respond to parathyroid extract or to be cured by infusion of spleen or bone marrow cells from normal littermates. *Am. J. Anat. 149*: 289-297.

MARKS, S.C. Jr. (1997). The structural basis for bone cell biology. *Acta Med. Dent. Helv. 107*: 141-157.

MARKS, S.C. Jr. (1998). The structural and developmental contexts of skeletal injury. Chapter 1. In *Diagnostic Imaging of Child Abuse*, (Ed. P.K. Kleinman). second edition, Mosby Inc., Philadelphia, PA. pp.2-7.

MARKS, S.C. Jr., LUNDMARK, C., WURTZ, T., ODGREN, P.R., MACKAY, C.A., MASON-SAVAS, A. and POPOFF, S.N. (1999). Facial development and type III collagen RNA expression: concurrent repression in the osteopetrotic (toothless, *tl*) rat and rescue after treatment with colony-stimulating factor-1. *Dev. Dyn. 215:* 117-125.

MARKS, S.C. Jr., MACKAY, C.A., JACKSON, M.E., LARSON, E.K., CIELINSKI, M.J., STANLEY, E.R. and AUKERMAN, S.L. (1993). The skeletal effects of colony-stimulating factor-1 in toothless (osteopetrotic) rats: persistent metaphyseal sclerosis and the failure to restore the subepiphyseal osteoclasts. *Bone 14*: 675-680.

MARKS, S.C. Jr., WOJTOWICZ, A., SZPERL, M., URBANOWSKA, E., MACKAY, C.A, WIKTOR-JEDRZEJCZAK, W., STANLEY, E.R. and AUKERMAN, S.L. (1992). Administration of colony stimulating factor 1 corrects some macrophage, dental and skeletal defects in an osteopetrotic mutation (toothless, *tl*) in the rat. *Bone 13*: 89-93.

NIYIBIZI, C., VISCONTI, C.S., GIBSON, G. and KAVALKOVICH, K. (1996). Identification and immunolocalization of type X collagen at the ligament-bone interface. *Biochem. Biophys. Res. Commun. 222*: 584-589.

NURMINSKAYA, M., MAGEE, C., NURMINSKY, D. and LINSENMAYER, T.F. (1998). Plasma transglutaminase in hypertrophic chondrocytes: expression and cell-specific intracellular activation produce cell death and externalization. *J. Cell Biol. 142*: 1135-1144.

ODGREN, P.R., POPOFF, S.N., SAFADI, F.F., MACKAY, C.A., MASON-SAVAS, A., SEIFERT, M.F. and MARKS, S.C. Jr. (1999). The toothless osteopetrotic rat has a normal vitamin D binding protein-macrophage activating factor (DBP-MAF) cascade and chondrodysplasia resistant to treatments with colony stimulating factor-1 (CSF-1) and/or DBP-MAF. *Bone 25*: 175-181.

OLSEN, B.R. (1995). Mutations in collagen genes resulting in metaphyseal and epiphyseal dysplasias. *Bone 17*: 45S-49S.

PHILBRICK, W.M. (1998). Parathyroid hormone-related protein is a developmental regulatory molecule. *Eur. J. Oral Sci. 106*: 32-37.

PHILBRICK, W.M., WYSOLMERSKI, J.J., GALBRAITH, S., HOLT, E, ORLOFF, J.J., YANG, K.H., VASAVADA, R.C., WIER, E.C., BROADUS, A.E. and STEWART, A.F. (1996). Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiological Rev. 76*: 127-173.

POKHAREL, R.K., ALIMSARDJONO, H., UNO, K., FUJII, S., SHIBA, R. and MATSUO, M. (1995). A novel mutation substituting tryptophan with arginine in the caboxyl-terminal, non-collagenous domain of collagen X in a case of Schmid metaphyseal chondrodysplasia. *Biochem. Biophys. Res. Commun. 217*: 1157-1162.

POPOFF, S.N. and MARKS, S.C. Jr. (1995). The heterogeneity of the osteopetroses reflect the diversity of cellular influences during skeletal development. *Bone 17*: 437-445.

REICHENBERGER, E., AIGNER, T., VON DER MARK, K., STRÖSS, H. and BERTLING, W. (1991). *In situ* hybridization studies on the expression of type X collagen in fetal human cartilage. *Dev. Biol. 148*: 562-572.

ROSATI, R., HORAN, G.S.B., PINERO, G.J., GAROFALO, S., KEENE, D.R., HORTON, W.A., VUORIO, E., DE CROMBRUGGHE, B. and BEHRINGER, R.R. (1994). Normal long bone growth and development in type X collagen null mice. *Nature Genet. 8*: 129-135.

SCHMID, T.M. and LINSENMAYER, T.F. (1990). Immunoelectron microscopy of type X collagen: supramolecular forms within embryonic chick cartilage. *Dev. Biol. 138*: 53-62.

SCHMID, T.M., BONEN, D.K., LUCHENE, L. and LINSENMAYER, T.F. (1991). Late events in chondrocyte differentiation: hypertrophy, type X collagen synthesis and matrix calcification. *In vivo 5*: 533-540.

SCHNEIDER, G.B., CUÉNOUD, M.L. and MARKS, S.C. Jr. (1979). The diagnosis and cure of neonatal osteopetrosis: experimental evidence from congenitally osteopetrotic *(ia)* rats. *Metab. Bone Dis. Relat. Res. 1*: 335-339.

SEIFERT, M.F. (1994). Lack of evidence for rickets in the osteopetrotic rat mutation, toothless. *J. Bone Miner. Res. 9*: 1813-1821.

SEIFERT, M.F. (1996). Abnormalities in bone cell function and endochondral ossification in the osteopetrotic toothless rat. *Bone 19*: 329-338.

SEIFERT, M.F., POPOFF, S.N. and MARKS, S.C. Jr. (1988). Skeletal biology in the toothless (osteopetrotic) rat. *Am. J. Anat. 183*: 158-165.

SHALHOUB, V., JACKSON, M.E., LIAN, J.B., STEIN, G.S. and MARKS, S.C. Jr. (1991). Gene expression during skeletal development in three osteopetrotic rat mutations: evidence for osteoblast abnormalities. *J. Biol. Chem. 266*: 9847-9856.

SIRES, U.I., SCHMID, T.M., FLISZAR, C.J., WANG, Z-Q. and GLUCK, S.L. (1995). Complete degradation of type X collagen requires the combined action of interstitial collagenase and osteoclast-derived cathepsin-B. *J. Clin. Invest. 95*: 2089-2095.

SUNDQUIST, K.T., JACKSON, M.E., HERMEY, D.C. and MARKS, S.C. Jr. (1995). Osteoblasts from the toothless (osteopetrotic) mutation in the rat are unable to direct bone resorption by normal osteoclasts in response to 1,25-dihydroxyvitamin D. *Tissue Cell 27*: 569-574.

VANKY, P., BROCKSTEDT, U., HJERPE, A. and WIKSTRÖM, B. (1998). Kinetic studies on epiphyseal growth cartilage in the normal mouse. *Bone 22*: 331-339.

VORTKAMP, A., LEE, K., LANSKI, B., SEGRE, G.V., KRONENBERG, H.M. and TABIN, C.J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science 273*: 613-622.

VORTKAMP, A., PATHI, S., PERETTI, G.M., CARUSO, E.M., ZALESKE, D.J. and TABIN, C.J. (1998). Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair. *Mech. Dev. 71*: 65-76.

VU, T.H., SHIPLEY, J.M., BERGERS, G., BERGER, J.E., HELMS, J.A., HANAHAN, D., SHAPIRO, S.D., SENIOR, R.M. and WERB, Z. (1998). MMP-9/Gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell 93*: 411-422.

WATANABE, H., KISLAUSKIS, E.H., MACKAY, C.A., MASON-SAVAS, A. and MARKS, S.C. Jr. (1998). Actin mRNA isoforms are differentially sorted in normal osteoblasts and sorting is altered in osteoblasts from a skeletal mutation in the rat. *J. Cell Sci. 111*: 1287-1292.

WATANABE, H., MACKAY, C.A., KISLAUSKIS, E., MASON-SAVAS, A. and MARKS, S.C. Jr. (1997). Ultrastructural evidence of abnormally short and maldistributed actin stress fibers in osteopetrotic (toothless) rat osteoblasts *in situ* after detergent perfusion. *Tissue Cell 29*: 89-98.

WEIR, E.C., PHILBRICK, W.M., AMLING, M., NEFF, L.A., BARON, R. and BROADUS, A.E. (1996). Targeted overexpression of parathyroid hormone-related peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Proc. Natl. Acad. Sci. USA 93*: 10240-10245.

WISNER-LYNCH, L.A., SHALHOUB, V. and MARKS, S.C. Jr., (1995). Administration of colony stimulating factor-1 (CSF-1) to toothless *(tl)* osteopetrotic rats normalizes osteoblast, but not osteoclast, gene expression. *Bone 16*: 611-618.

WURTZ, T., BRANDSTEN, C. LUNDMARK, C. and CHRISTERSSON, C. (1998). Collagen mRNA expression during tissue development: the temporospacial order coordinates bone morphogenesis with collagen fiber formation. *Matrix Biol. 17*: 349-360.

ZEREGA, B., CERMELLI, S., BIANCO, P., CANCEDDA, R. and CACEDDA, F.D. (1999). Parathyroid hormone [PTH(1-34)] and parathyroid hormone-related protein [PTHrP(1-34)] promote reversion of hypertrophic chondrocytes to a prehypertrophic proliferating phenotype and prevent terminal differentiation of osteoblast-like cells. *J. Bone Miner. Res. 14*: 1281-1289.

ZOU, H., WIESER, R., MASSAGUÉ, J. and NISWANDER, L. (1997). Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev. 11*: 2191-2203.

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