

# Increased Expression of TGF- $\beta$ 2 in Osteoblasts Results in an Osteoporosis-like Phenotype

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**Abstract.** The development of the skeleton requires the coordinated activities of bone-forming osteoblasts and bone-resorbing osteoclasts. The activities of these two cell types are likely to be regulated by TGF- $\beta$ , which is abundant in bone matrix. We have used transgenic mice to evaluate the role of TGF- $\beta$ 2 in bone development and turnover. Osteoblast-specific overexpression of TGF- $\beta$ 2 from the osteocalcin promoter resulted in progressive bone loss associated with increases in osteoblastic matrix deposition and osteoclastic bone resorption. This phenotype closely resembles

the bone abnormalities seen in human hyperparathyroidism and osteoporosis. Furthermore, a high level of TGF- $\beta$ 2 overexpression resulted in defective bone mineralization and severe hypoplasia of the clavicles, a hallmark of the developmental disease cleidocranial dysplasia. Our results suggest that TGF- $\beta$ 2 functions as a local positive regulator of bone remodeling and that alterations in TGF- $\beta$ 2 synthesis by bone cells, or in their responsiveness to TGF- $\beta$ 2, may contribute to the pathogenesis of metabolic bone disease.

**N**ORMAL skeletal morphogenesis relies upon the coordinated activities of multiple cell types. The principal cell types in bone are osteoblasts, which are of mesenchymal origin and synthesize bone matrix, and osteoclasts, which are derived from the hematopoietic system and resorb bone. During development and adult life, osteoblasts and osteoclasts continuously replace old bone with new bone through a process termed bone remodeling (for a review see Parfitt, 1994). Bone remodeling occurs through discrete cycles of localized resorption followed by new bone synthesis in resorbed areas. The coordinated nature of these cycles implies the existence of mechanisms that couple osteoblastic bone formation to osteoclastic bone resorption in a site-specific manner and link the remodeling cycle to the local differentiation of osteoblasts and osteoclasts from their respective precursor populations. By matching resorption with formation, bone remodeling normally maintains skeletal integrity and preserves bone mass and shape.

The failure to coordinate osteoclastic bone resorption with osteoblastic bone deposition results in metabolic bone disease. In many cases, such as osteoporosis and hyperparathyroidism, this failure leads to a progressive net loss of bone mass with time. The nature of the bone re-

modeling defects observed in various bone diseases suggests that distinct components of the remodeling cycle, including the frequency and extent of osteoclastic bone resorption, as well as the rate of osteoblastic bone deposition, are actively regulated (Parfitt, 1983). Several systemic hormones are known to influence bone turnover, and it is likely that they act through local factors present within bone (Mundy, 1993). How these factors control bone development and remodeling by locally regulating the activity, differentiation, and interactions of resident bone cells, is largely unknown.

Several lines of evidence have suggested that the secreted polypeptide transforming growth factor- $\beta$  (TGF- $\beta$ ) could be involved in the local regulation of skeletal development and turnover (for a review see Centrella et al., 1994). High concentrations of all three TGF- $\beta$  isoforms (TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3) can be extracted from mineralized bone matrix, and TGF- $\beta$  is synthesized by osteoblasts and osteoclasts in vivo (Seyedin et al., 1985; Robey et al., 1987; Sandberg et al., 1988; Pelton et al., 1991). TGF- $\beta$  has potent effects on bone cells in vitro and in vivo. Local injection of TGF- $\beta$  under the periosteum stimulates cartilage and bone formation (Noda and Camilliere, 1989; Joyce et al., 1990), and systemic injection of TGF- $\beta$ 2 leads to a generalized increase in osteoblastic activity (Rosen et al., 1994). In vitro, TGF- $\beta$  induces extracellular matrix secretion by osteoblasts, inhibits matrix mineralization, and modulates osteoprogenitor cell proliferation and the expression of osteoblastic differentiation

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markers (for reviews see Bonewald and Dallas, 1994; Centrella et al., 1994). Results, however, have varied substantially depending upon the cell system. The effects of TGF- $\beta$  on osteoclast differentiation and bone resorption are also complex and may be indirectly modulated through nonosteoclastic cell types (Hattersley and Chambers, 1991). Neonatal and fetal organ culture studies have generally indicated that TGF- $\beta$  inhibits osteoclast differentiation from bone marrow monocytes, yet stimulates bone resorption by differentiated osteoclasts (Tashjian et al., 1985; Chenu et al., 1988; Pfeilschifter et al., 1988; Dieudonné et al., 1991; Hattersley and Chambers, 1991).

The normal role of TGF- $\beta$  in bone development has been hard to infer from these studies. Besides its complex and variable effects on bone cell populations *in vitro* and *in vivo*, a given experimental result with TGF- $\beta$  may be potentially relevant to many different aspects of skeletal morphogenesis, including the generation of bone shape, bone growth, or bone remodeling. Furthermore, the exogenous application of TGF- $\beta$  does not mimic its route of production within bone. Osteoblasts produce TGF- $\beta$  largely as a bone matrix-bound latent complex that may be unable to induce cellular responses unless first released from mineralized bone during osteoclastic bone resorption (Pfeilschifter and Mundy, 1987). Lastly, the short term nature of TGF- $\beta$  administration in both *in vitro* and *in vivo* experiments precludes an evaluation of the skeletal actions of TGF- $\beta$  at steady state.

To gain more direct insight into the role of TGF- $\beta$  in bone morphogenesis, we generated transgenic mice that overexpress active TGF- $\beta$ 2 specifically in bone. Increased synthesis of TGF- $\beta$ 2 in osteoblasts leads to a dramatic, age-dependent loss of bone mass. This phenotype is attributed to a primary defect in bone remodeling, associated with increased activities of both osteoblasts and osteoclasts. Our results implicate TGF- $\beta$  and more specifically TGF- $\beta$ 2 as an important local regulator of bone remodeling *in vivo* and raise the possibility that altered TGF- $\beta$ 2 expression or responsiveness may underlie the local pathogenesis of osteoporosis, hyperparathyroidism, and other metabolic bone diseases.

## Materials and Methods

### Expression Plasmid

PCR mutagenesis was performed on the human TGF- $\beta$ 2 cDNA (de Martin et al., 1987) to replace cysteine codons 226 and 228 in the TGF- $\beta$ 2 precursor with codons TCT and AGC for serine, and to introduce a 5' EcoRI site (preceding nucleotide 178) and a 3' BamHI site (after nucleotide 1426) to allow subcloning. The resulting fragment was sequenced to confirm that no other point mutations were introduced during PCR amplification. The expression plasmid pOc- $\beta$ 2C2S2, cloned into pcDNAII (Invitrogen, San Diego, CA), consisted of a 1.8-kb fragment of the rat osteocalcin promoter (Baker et al., 1992), followed by a 0.65-kb fragment spanning the rabbit  $\beta$ -globin second intron (O'Hare et al., 1981), the mutated 1.3-kb TGF- $\beta$ 2 cDNA fragment and a 0.63-kb fragment of the human growth hormone gene to allow polyadenylation. Mature human TGF- $\beta$ 2 expressed by pOc- $\beta$ 2C2S2 has 97% amino acid sequence identity to mouse TGF- $\beta$ 2 (Miller et al., 1989). To verify that this plasmid could express TGF- $\beta$ 2, it was transfected into ROS 17/2.8 osteosarcoma cells using the Ca<sub>2</sub>PO<sub>4</sub> precipitation method. As a control, cells were transfected with a plasmid expressing  $\beta$ -galactosidase under control of the osteocalcin promoter. Tissue culture supernatant was collected 72 h after transfection,

concentrated 10-fold on a Centricon C-10 column (Amicon, Inc., Beverly, MA), and assayed by TGF- $\beta$ 2 ELISA (Amersham Corp., Arlington Heights, IL).

### Transgenic Mice

DBA2  $\times$  C57BL/6 F1 (B6D2 F1) mice (Jackson Laboratories, West Grove, PA) were used to generate transgenic mice using standard techniques (Hogan et al., 1986). We injected the osteocalcin-TGF- $\beta$ 2 expression insert excised as a 4.4-kb BamHI fragment from pOc- $\beta$ 2C2S2, and purified by GeneClean (Bio 101). Transgenic mice were identified using Southern blot analysis of tail DNA (Laird et al., 1991) using the  $\beta$ -globin intron fragment as a hybridization probe. Transgenic lines were established and maintained on a B6D2 F1 background, and subsequent generations of transgenic animals were identified by nonradioactive dot blot using either the  $\beta$ -globin intron or growth hormone poly(A) fragments as probes. Nonradioactive hybridization reagents were purchased from Boehringer Mannheim (Indianapolis, IN) and used as described (Engler et al., 1993).

### RNA Analysis

Total bone RNA was isolated from 4–5 wk-old animals. Femurs and humeri were dissected and cleaned of soft tissues. Epiphyses were cut off, and the bone marrow was flushed out with PBS. Bones were then crushed in a mortar in liquid nitrogen, and further homogenized on ice in an Omni 2000 tissue homogenizer (Omni International, Marietta, GA) for 30 s in 4 ml Ultraspec RNA isolation reagent (Biotecx, Houston, TX). RNA was purified according to the manufacturer's instructions, and treated with RQ1 DNAase (Promega, Madison, WI) before further analysis. One mouse typically yielded 25–40  $\mu$ g total bone RNA.

Total bone RNA from two mice from each transgenic line was analyzed for the presence of spliced transgene mRNA by reverse-transcriptase PCR (RT-PCR). Reverse-transcriptase (RT)<sup>1</sup> reactions were performed on 500–800 ng RNA in 20  $\mu$ l of 1 $\times$  PCR buffer (GIBCO-BRL, Gaithersburg, MD), 2.5 mM MgCl<sub>2</sub>, 1 mM dNTPs, 100 pmol random hexanucleotides (Pharmacia LKB Biotechnology, Piscataway, NJ), 1 mM DTT, 0.5  $\mu$ l RNasin (Promega), and 1  $\mu$ l MMTV reverse transcriptase (GIBCO-BRL). Control reactions without reverse transcriptase were done in parallel. RT reactions were incubated for 10 min at room temperature, 30 min at 37°C, 30 min at 42°C, and then stored directly at 4°C. To detect the spliced transgene cDNA by PCR, the 5' primer (5'-TCCTGAGAACTTCAGG/CTCCTG-3') spanned the rabbit  $\beta$ -globin intron splices sites and the 3' primer (5'-CCTTGGCGTAGTACTCTTCGTC-3') was located within the TGF- $\beta$ 2 cDNA, to yield an expected product of 378 bp. 1  $\mu$ l of RT reaction was amplified by PCR cycling in a 50- $\mu$ l reaction volume containing 1 $\times$  PCR buffer (GIBCO-BRL), 3 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.1% Triton X-100, 10 pmol each primer, and 0.25  $\mu$ l Taq polymerase (GIBCO-BRL). 20  $\mu$ l of the reaction was run on a 2% TBE agarose gel stained with ethidium bromide. To confirm that the resulting DNA bands corresponded to TGF- $\beta$ 2 cDNA, we performed Southern blot hybridization using a human TGF- $\beta$ 2 cDNA fragment as probe. As a control for RNA quality and successful RT reactions, 1  $\mu$ l of each RT reaction was amplified using primers specific for the osteocalcin gene (Araki et al., 1993) in 1.5 mM MgCl<sub>2</sub>.

### Preparation of Bone Extracts

Bone extracts were prepared as described (Finkelman et al., 1991) with modifications, from 35-d-old transgenic and nontransgenic females. Femurs and humeri were cleaned of soft tissue and the epiphyses and bone marrow were removed. Bones were stored at -70°C. To prepare bone powder, bones were crushed in a mortar in liquid nitrogen and fat was removed as described (Finkelman et al., 1991), yielding 15–30 mg bone residue. Bone powder was then extracted for 36 h at 4°C in 300  $\mu$ l extraction buffer (4 M guanidine hydrochloride, 10% EDTA, 30 mM Tris-HCl pH 7.4) containing 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, and 1 mM PMSF, with constant rotation. After removal of the debris by 15 min centrifuga-

1. *Abbreviations used in this paper:* Ccd, cleidocranial dysplasia; PTH, parathyroid hormone; RT, reverse-transcriptase.

tion, supernatants were collected and stored at 4°C and assayed for protein concentration using the Bradford assay (Biorad Labs., Hercules, CA).

### **Determination of TGF- $\beta$ Protein Concentrations**

TGF- $\beta$ 1 and TGF- $\beta$ 2 concentrations were quantitated using TGF- $\beta$ 1- and TGF- $\beta$ 2-specific ELISA kits (R&D Systems, Minneapolis, MN and Amersham Corp., respectively), according to the manufacturers' instructions. All bone samples were assayed in duplicate.

In bone samples, TGF- $\beta$  was already activated due to the protein extraction in guanidine-HCl and did not require further acid-activation before ELISA. However, to assay TGF- $\beta$  in culture media and plasma, samples were acid-activated (Lawrence et al., 1985) by an addition of 1/10th vol of 1.2 N HCl at room temperature and neutralized after 20 min by addition of 1/5th vol 0.72 N NaOH, 0.5 M Hepes. 80  $\mu$ l of plasma were assayed per mouse, with two mice per test group, and five control mice. Active and latent TGF- $\beta$ 2 levels in cell culture media were assayed using duplicate samples either treated or untreated with acid.

### **Serology, Body Weights**

Serum was collected by eye bleeds from 3 sex-matched transgenic and 3 nontransgenic littermates per age group. Calcium, phosphorous, total protein, and creatinine were determined by automated protocols (California Veterinary Diagnostics, Inc., West Sacramento, CA). Heparinized tubes were used for the collection of plasma. Weights of juvenile mice were measured from several groups of 2-3 transgenic and nontransgenic sex-matched littermates.

### **X-ray Analysis**

After sacrifice by CO<sub>2</sub> inhalation, radiographs were taken on a faxitron model 43805 N (Hewlett-Packard, Palo Alto, CA), using Kodak X-OMAT TL film. Exposure was for 1 min at 35 kVp. For 35-d-old mice, a total of 16 D4, 7 D5 mice, and 13 control littermates were analyzed. For 5-11-month-old males and virgin females, a total of 7 D4 and 8 D5 mice, as well as 20 control littermates and nonlittermates were analyzed. Consistent results were obtained from mice of the same line and age.

### **Histology and TGF- $\beta$ 2 Immunohistochemistry**

For paraffin-embedded sections, bones were fixed at 4°C in 2% paraformaldehyde/PBS, and decalcified for 4 h in 22.5% formic acid, 10% sodium citrate at room temperature. 5  $\mu$ m sections were stained with hematoxylin and eosin for routine analysis using established procedures (Sheehan and Hrapchak, 1980). For each age group, 2-3 bones per mouse from at least 2-5 mice of each line were analyzed. Serial cross sections of tibia were standardized in reference to the hypertrophic cartilage-metaphyseal junction, defined as the cross section with an area occupied by 50% hypertrophic cartilage and 50% primary spongiosa.

Anti-TGF- $\beta$ 2 immunohistochemistry was performed as modified from Pelton et al. (1991) using rabbit anti-TGF- $\beta$ 2 (kindly supplied by Dr. Leslie Gold, New York University Medical School) at a concentration of 2.5  $\mu$ g/ml. An HRP-conjugated donkey anti-rabbit secondary antibody (Amersham) was used at a 1:100 dilution. Diaminobenzidine was used as substrate for the HRP reaction and the sections were counterstained with hematoxylin.

For osteoclast detection using histochemical staining for tartrate-resistant acid phosphatase (TRAP) activity, femurs fixed in 2% paraformaldehyde/PBS were decalcified for 3 d at 4°C in 10% EDTA, 0.1 M Tris, pH 6.95, embedded in Tissue-Tek OCT compound, and sectioned at 10  $\mu$ m. After equilibration in 0.2 M sodium acetate, 50 mM sodium tartrate, pH 5.0, for 20 min at room temperature, sections were incubated for 17 min at 37°C in the same buffer containing 0.5 mg/ml naphthol AS-MX phosphate (Sigma Chem. Co., St. Louis, MO) and 1.1 mg/ml Fast Red Violet LB salt (Sigma). Sections were counterstained in methyl green.

To detect bone mineralization, 4  $\mu$ m undecalcified longitudinal sections of the tibia (see below) were stained by the von Kossa technique (Sheehan and Hrapchak, 1980) and counterstained with toluidine blue.

### **Quantitative and Kinetic Analyses**

Trabecular surface density was calculated by a stereological method using transverse lines superimposed upon bone section micrographs (Weibel,

1969). From longitudinal sections of the proximal tibial metaphysis, data were collected from the entire area of the bone marrow cavity 200-1,800  $\mu$ m from the growth plate for 35-d-old mice, and 0-1,600  $\mu$ m from the growth plate for 7-11-month-old mice. Values represent the mean  $\pm$  standard deviation of the surface density calculated from at least three non-consecutive sections per mouse from 3 D4, 2 D5, and 3 nontransgenic littermate 35-d-old mice, and 2 D4, 2 D5, and 4 control 7-11-month-old mice.

Data for osteocyte density, and osteoblast and osteoprogenitor cell number were taken from cortical bone cross sections of the proximal tibia, 2.5-3.1 mm from the growth plate. Osteocyte density was calculated as the 3:2 power of the two-dimensional density in cross section, and osteoblast and osteoprogenitor cell number was calculated as the square of the one-dimensional density on the endosteal surface. Values represent the mean  $\pm$  standard deviation of 30-70 measurements per group of 2 D4 and 3 littermate control mice. Standard deviations maintain the same percent error of the original lower-dimensional value. The rate of osteocyte differentiation was calculated as the product of the osteocyte density and the bone volume produced per day in a mm<sup>2</sup> unit area (the mineral apposition rate multiplied by the mm<sup>2</sup> unit area).

For the kinetic analyses, 31 day sex-matched transgenic and nontransgenic littermates were given intraperitoneal injections of 2.5 mg/kg calcitonin, followed by similar injections of 25 mg/kg tetracycline 3 d later. Mice were sacrificed at day 35, and their bones were fixed in 70% ethanol at 4°C. Before processing for undecalcified sectioning, bones were prestained using the Villanueva bone stain (osteochrome stain, Polysciences Inc., Niles, IL) for 3 d at 4°C. Matched bones were dehydrated and embedded with identical orientation in methylmethacrylate, sectioned at 4  $\mu$ m, and viewed under UV light.

Since there was virtually no trabeculation in bones from D4 animals, including the vertebrae, data for the mineral apposition rate and mineralization lag time was taken from epiphyseal and diaphyseal endosteal surfaces of the tibia. Individual measurements for the mineral apposition rate were calculated as the distance between the double fluorochrome labels divided by 3 d, and for the mineralization lag time as the osteoid seam width divided by the mineral apposition rate at the same location. Values represent the mean  $\pm$  standard deviation of 40-80 measurements per group of 2 D4 and 2 littermate control mice.

### **Cleared Skeletal Preparations**

Cleared skeletons stained with alizarin red or alcian blue staining were prepared as adapted from Kaufman (1992). We analyzed four litters of D4 male  $\times$  B6D2 F1 female crosses, giving a total of 19 transgenic and 16 nontransgenic embryonic day 15.5 and 2-d-old offspring.

## **Results**

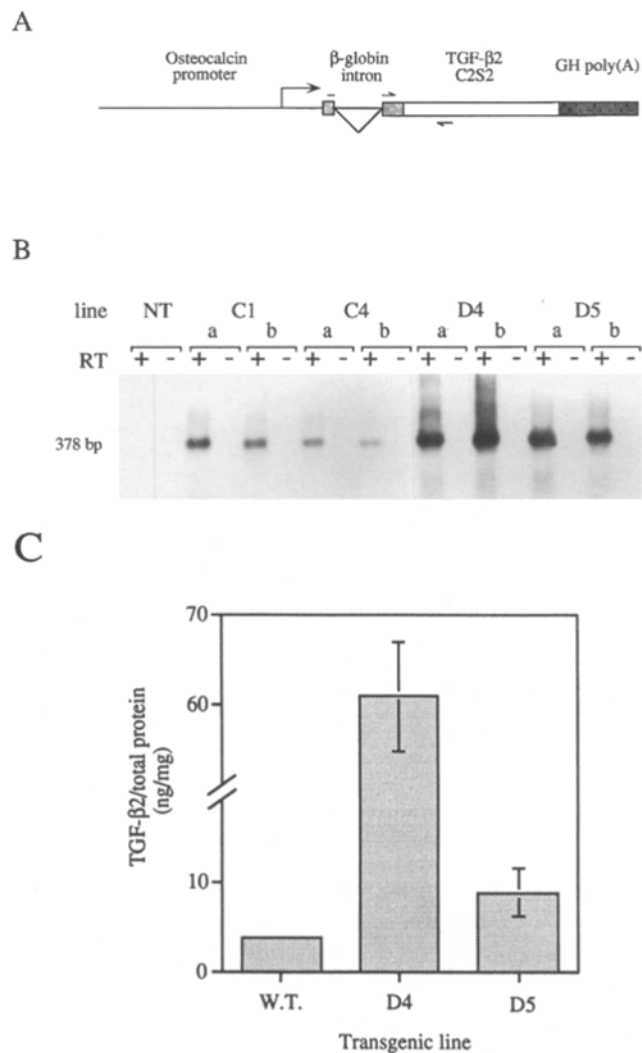
### **TGF- $\beta$ 2 Expression under the Control of the Osteocalcin Promoter**

We designed the plasmid pOc- $\beta$ 2C2S2 to drive the osteoblast-specific expression of biologically active TGF- $\beta$ 2 in transgenic mice (Fig. 1 a). Among the three isoforms of TGF- $\beta$ , we elected to express TGF- $\beta$ 2 because it is normally expressed by osteoblasts and is thought to be more active than TGF- $\beta$ 1 in inducing cartilage and bone formation (Seyedin et al., 1985; Joyce et al., 1990). TGF- $\beta$  is normally made in a latent or biologically inactive form due to noncovalent interactions between its large precursor segments and the mature TGF- $\beta$  homodimer (Centrella et al., 1994). To assure the secretion and availability of active TGF- $\beta$ 2, we introduced two point mutations that resulted in the replacement of cysteines at positions 226 and 228 in the TGF- $\beta$ 2 precursor segment by serines. Similar mutations of the corresponding cysteines in the TGF- $\beta$ 1 precursor (at positions 233 and 235) have been shown to lead to the spontaneous activation of a fraction of the TGF- $\beta$  secreted by transfected cells in culture, presumably as a re-

sult of destabilization of the interactions between the precursor segments and the active TGF- $\beta$  dimer (Brunner et al., 1989).

To achieve specific transgene expression in bone, we used a 1.8-kbp fragment of the rat osteocalcin promoter previously shown to direct osteoblast-specific expression (Baker et al., 1992). Osteocalcin is an abundant bone ma-

trix protein specifically expressed by mature osteoblasts, but not by osteoprogenitor cells (Bronckers et al., 1985; Groot et al., 1986). We verified the expression of TGF- $\beta$ 2 from pOc- $\beta$ 2C2S2 after transfection of the plasmid into the osteocalcin-expressing osteosarcoma cell line ROS 17/2.8. The double cysteine to serine mutation caused spontaneous activation of  $\sim$ 10% of the total amount of secreted TGF- $\beta$ 2 (data not shown).



**Figure 1.** Generation and biochemical characterization of transgenic mice. (A) Schematic presentation of the plasmid pOc- $\beta$ 2C2S2 expressing the human TGF- $\beta$ 2 with cysteines at positions 226 and 228 within the precursor segment replaced by serines. The direction of transcription and the position of the primers for RT-PCR analysis are indicated. (B) RT-PCR analysis of transgene mRNA expression from bone RNA. The RT-PCR generated cDNA fragment was subjected to Southern blot analysis using a human TGF- $\beta$ 2 cDNA probe. The four lines that express the transgene mRNA are shown, and *a* and *b* represent two different mice of each line. Abbreviations: NT, nontransgenic; RT, reverse-transcriptase. (C) Quantitation of TGF- $\beta$ 2 levels in bone matrix of 35-d-old female mice. TGF- $\beta$ 2 concentrations were measured by TGF- $\beta$ 2 ELISA and normalized to total extracted protein concentration. The graph represents the mean and standard deviations of duplicate measurements of two animals per transgenic line and four nontransgenic littermates.

### Transgenic Mice Overexpressing TGF- $\beta$ 2 in Osteoblasts

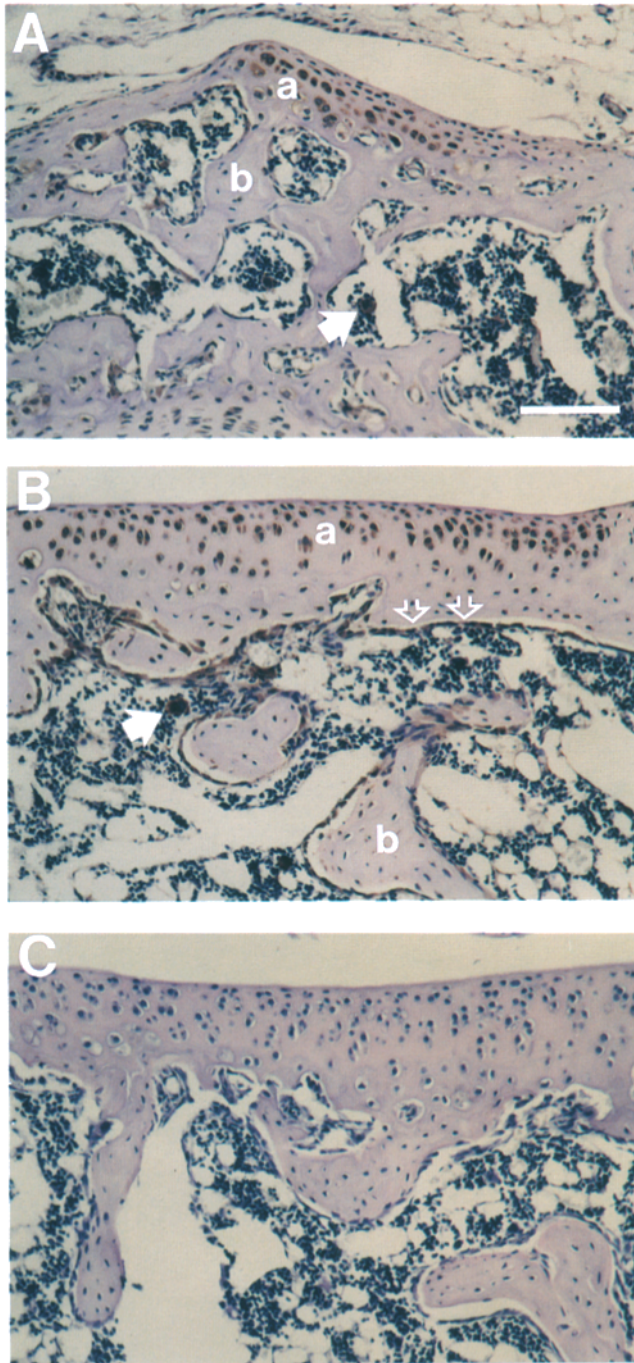
We established eight independent transgenic lines in which the transgene of pOc- $\beta$ 2C2S2 was stably integrated into the genome (data not shown). Four of these lines expressed the transgene mRNA as evaluated by RT-PCR of bone mRNA (Fig. 1 *b*). The two mouse lines with the highest mRNA levels, D4 and D5, were chosen for detailed characterization. In contrast to the D5 line, we were unable to generate homozygous D4 animals, presumably as a result of intrauterine or perinatal death. We assessed transgene expression at the protein level using a specific ELISA to quantitate the TGF- $\beta$ 2 extractable from bone. Bone powder extracts from hemizygous D4 and D5 mice contained 16- and 2.5-fold more TGF- $\beta$ 2 than bone extracts from nontransgenic mice, as normalized to total extracted protein (Fig. 1 *c*). The use of guanidine hydrochloride, which activates TGF- $\beta$ 2 in this extraction procedure, did not allow us to determine what fraction of the extracted TGF- $\beta$ 2 was active in situ. A TGF- $\beta$ 1-specific ELISA revealed that control bones contained 7.5-fold more TGF- $\beta$ 1 than TGF- $\beta$ 2 (data not shown). The level of TGF- $\beta$ 1 was unaltered in extracts of D5 bones, but was  $\sim$ 40% lower in D4 bones.

To localize the cellular sites of synthesis of TGF- $\beta$ 2 within bone, we stained longitudinal sections of the tibia of 35-d-old D4 mice with a TGF- $\beta$ 2-specific antibody. Sections of transgenic bone showed a distinct increase in the TGF- $\beta$ 2 immunoreactivity of osteoblasts as compared with control sections (Fig. 2). The increased immunoreactivity was very pronounced in the osteoblasts lining epiphyseal bone surfaces (Fig. 2 *b*, open arrows), but not in osteoblasts within the metaphysis (data not shown). In accordance with the osteoblast-specific expression of the osteocalcin promoter, there was no increase in the low level of endogenous TGF- $\beta$ 2 staining in bone marrow cells. In addition, both transgenic and control sections showed similar levels of strong endogenous TGF- $\beta$ 2 immunostaining in megakaryocytes (Fig. 2, *a* and *b*, arrows), osteoclasts and articular chondrocytes, and moderate immunostaining in hypertrophic chondrocytes (Fig. 2 and data not shown).

### Macroscopic Evaluation of the Skeletal Phenotype

X-ray analysis revealed multiple skeletal defects in young mice of the high-expressing D4 line (Fig. 3). At 35 d of age, hemizygous D4 mice had a dramatic decrease in bone density (osteopenia) compared to normal littermates, as revealed by the generally radiolucent appearance of the entire skeleton (compare Fig. 3, *b* and *d* with *a* and *c*) and the dramatic reduction in trabecular bone particularly evident in the metaphyses of long bones (Fig. 3, *a* and *b*, arrows). Furthermore, the cortices of long bones were thin-





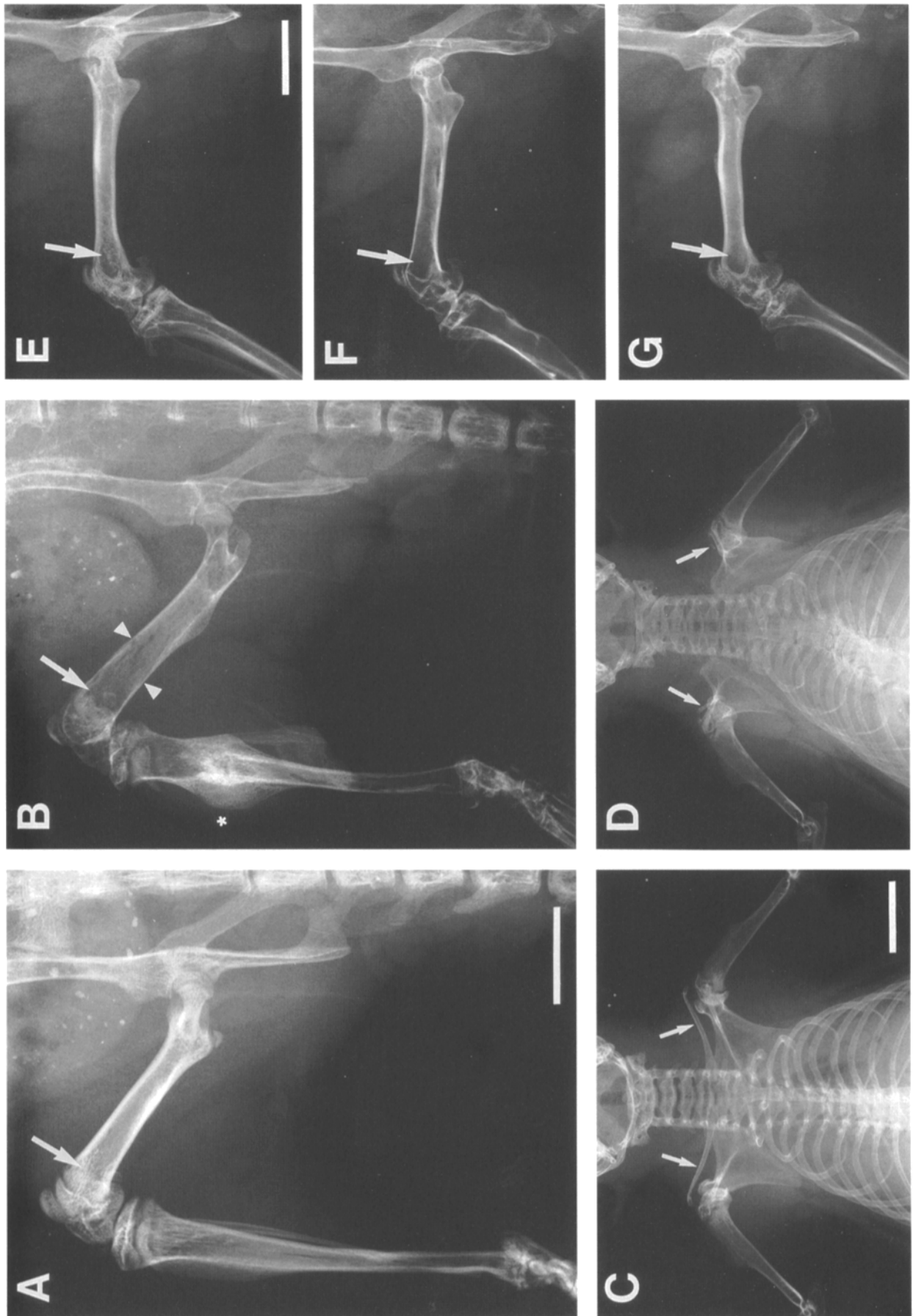
**Figure 2.** Immunohistochemical detection of TGF- $\beta$ 2 expression in bone. Sections are through the proximal epiphysis of the tibia from 35-d-old nontransgenic littermate (A) and D4 transgenic (B and C) females. C serves as a control of B in which the primary antibody against TGF- $\beta$ 2 is omitted from the staining procedure. The use of diaminobenzidine as a substrate results in brown staining for TGF- $\beta$ 2. Sections are counterstained with hematoxylin (blue). Transgenic bone shows strong TGF- $\beta$ 2 immunoreactivity in osteoblasts lining the endosteal surfaces (*open arrows*) in contrast to the low level of immunoreactivity within similar osteoblasts in nontransgenic bone (A). Endogenous TGF- $\beta$ 2 staining is seen in articular chondrocytes (*a*) and megakaryocytes (*closed arrows*). A low level of staining is seen in the bone marrow of both sections. Note also the increased number of plump, mature osteoblasts lining the surfaces of transgenic bone (B). Scale bar shows 100  $\mu$ m. Abbreviations: *a*, articular chondrocytes; *b*, bone.

ner than normal and frequently contained multiple internal radiolucent defects (Fig. 3 *b*, *arrowheads*). Fracture calluses were regularly observed in long bones (Fig. 3 *b*, *asterisk*), and callus-like focal lesions were frequently observed in the extremities, particularly at the distal ends of the radius, ulna, tibia, and fibula (data not shown). Based on their location and ultimate healing with time, these distal lesions most likely corresponded to stress fractures. The increased fragility of transgenic bones was also evident by the ease with which they were broken during dissection. By 7 months, hemizygous D4 mice were extremely osteopenic, with a glassy appearance of the entire skeleton (compare Fig. 3 *f* with 3 *e*), a virtual absence of trabecular bone inside long bones (*arrows*), and a strikingly lower level of trabecular bone in the vertebrae. Soft tissue calcifications were apparent in the necks of aging D4 mice (1-yr-old). Lastly, whereas all other bones had generally normal shapes and sizes, all D4 mice lacked normal clavicles and only small vestiges of their distal ends were apparent (Fig. 3, *c* and *d*, *arrows*). This aspect of the D4 phenotype will be described in more detail below.

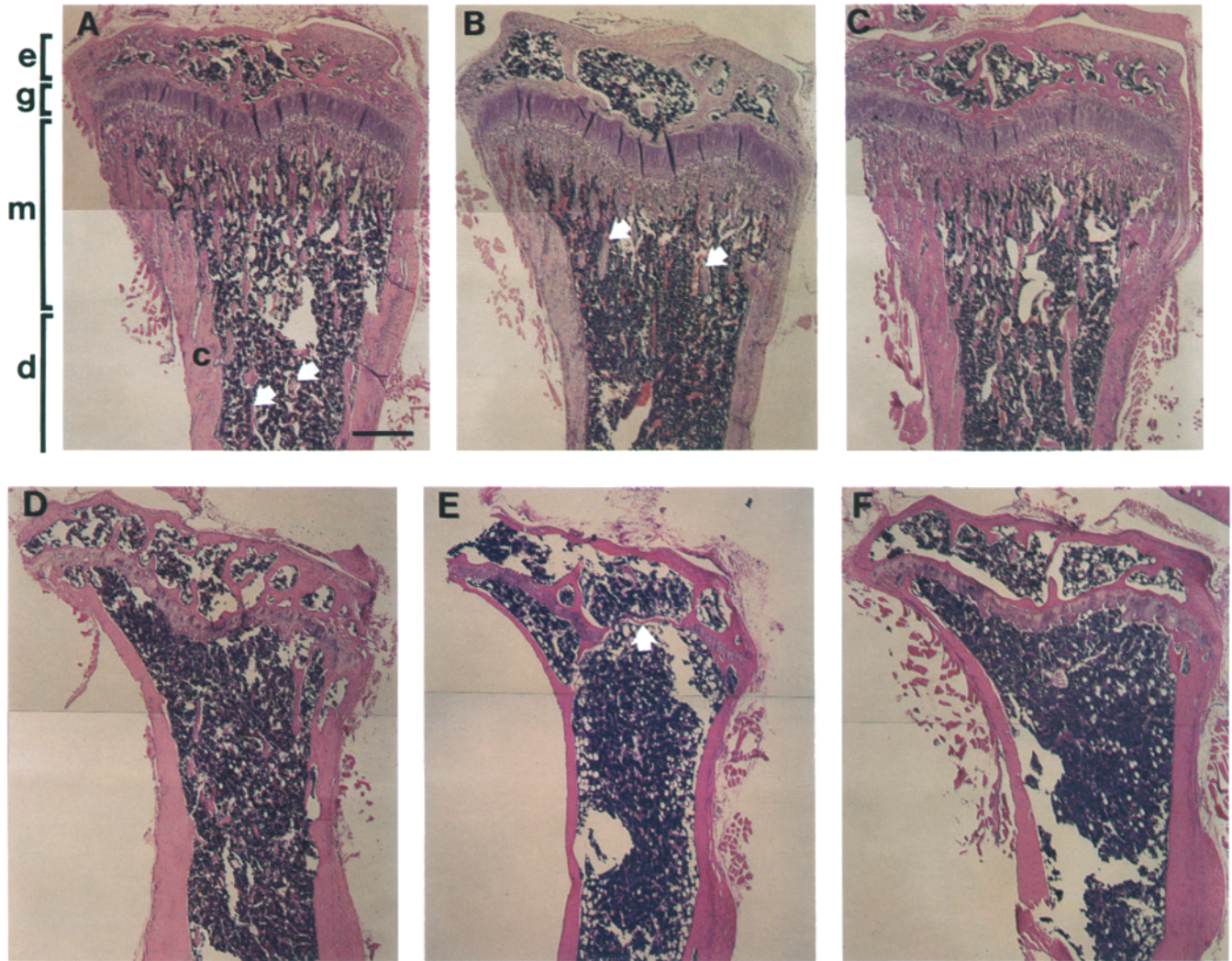
The D5 line had a considerably milder phenotype than the D4 line, consistent with its much lower level of TGF- $\beta$ 2 overexpression. Young (35 d) hemizygous and homozygous D5 mice did not have any obvious defects detectable by X-ray analysis; however, by 7 months of age, hemizygous D5 mice had a dramatic reduction of trabecular bone in the metaphyses of long bones (Fig. 3 *g*, *arrow*) and in the vertebral bodies, particularly of the lower thoracic and upper lumbar regions (data not shown). The skeletal abnormalities in both lines were similar in males and virgin females.

Aside from these abnormalities, transgenic mice appeared generally healthy. The mortality rate and fertility of transgenic animals were similar to wild type. All visceral organs appeared grossly normal at autopsy. 35-d-old mice had normal body weights, tooth eruption was not delayed, and teeth appeared normal. Importantly, the lengths of long bones, an indication of longitudinal growth, were also normal. Only with increasing age did the body weight of D4 mice decrease in comparison to normal littermates ( $\sim 60\%$  of normal in 6-month-old D4 mice).

Based on various blood serum tests, there was no indication of a general metabolic disease in the high overexpressing D4 line. No differences in serum calcium and phosphorus levels were apparent between 35-d-old transgenic and control mice, while 6-month-old transgenic animals had normal serum phosphorus, low serum calcium, and normal serum protein levels (data not shown). These results indicate that D4 mice did not have primary hyperparathyroidism or a humoral hypercalcemia syndrome, yet had a low circulating calcium level at older ages. Normal serum creatinine levels at 6 months of age excluded renal failure. Plasma TGF- $\beta$ 2 levels were normal in D5 mice, but were elevated 3–4-fold in 35-d-old D4 mice, to  $\sim 2$  ng/ml. At this level, circulating TGF- $\beta$  is not physiologically relevant since it is inactivated by the abundant serum protein  $\alpha$ 2-macroglobulin (O'Connor and Wakefield, 1987; Huang et al., 1988). Taken together, our analyses support the notion that the observed skeletal abnormalities resulted from the localized overexpression of TGF- $\beta$ 2 and not from a general endocrine disturbance. It is likely that



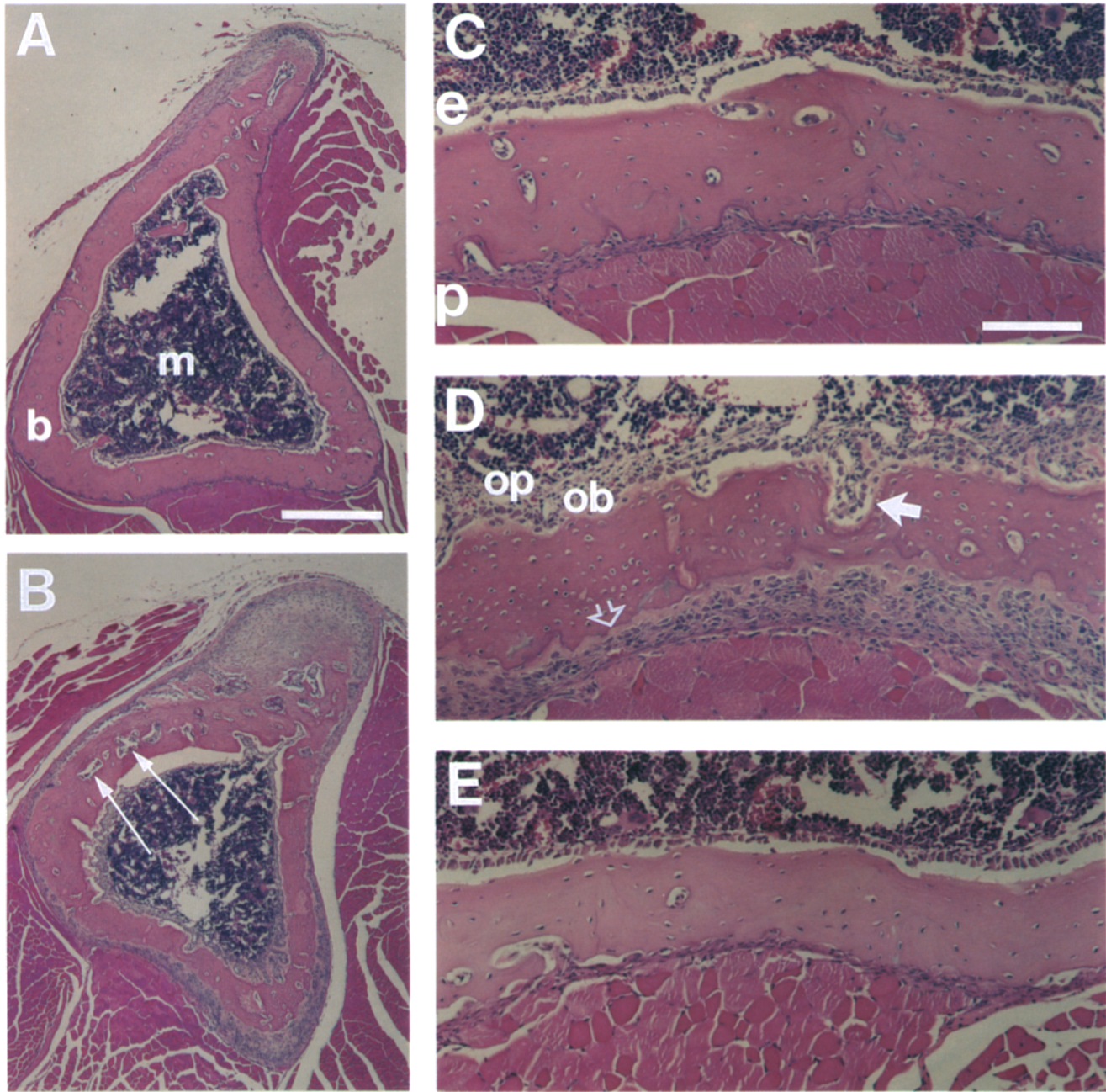




**Figure 4.** Age-dependent changes in histological appearance of transgenic bone. (A–C) Longitudinal sections through the proximal tibia of 35-d-old wild type (A), D4 (B), and homozygous D5 (C) males. As compared with nontransgenic bone (A), D4 bone shows a thinned cortex and reduced trabeculation, dramatically in the metaphysis and moderately in the epiphysis (B). In the metaphysis, transgenic trabecular bone is apparent only in the immediate area of the growth plate, whereas in nontransgenic bone it extends down the length of the bone marrow cavity towards the diaphysis (arrows). In contrast to D4 bone, D5 bone (C) appears histologically normal at this age. The epiphyseal growth plates in both the D4 and D5 sections appear normal. (D–F) Longitudinal sections through the proximal tibia of 7-month-old wild type (D), D4 (E), and hemizygous D5 (F) males. Cortical bone thinning and the extreme reduction in trabecular bone mass at this age is evident in D4 bone (E), as compared with nontransgenic bone (D), with a complete loss of trabeculation in the metaphysis and a dramatic reduction of trabeculation in the epiphysis. The residual growth plate cartilage is also more eroded in the D4 section (arrow) than in nontransgenic bone. In contrast to the normal phenotype in D5 bone at 35 d, 7-month-old bone shows a complete loss of metaphyseal trabeculation and a moderate reduction in epiphyseal trabeculation (F), although clearly not as severe as in D4 bone. Abbreviations: e, epiphysis; g, growth plate; m, metaphysis; d, diaphysis; c, cortical bone. Decalcified, paraffin-embedded sections were stained with hematoxylin and eosin. Scale bar shows 400  $\mu\text{m}$ .

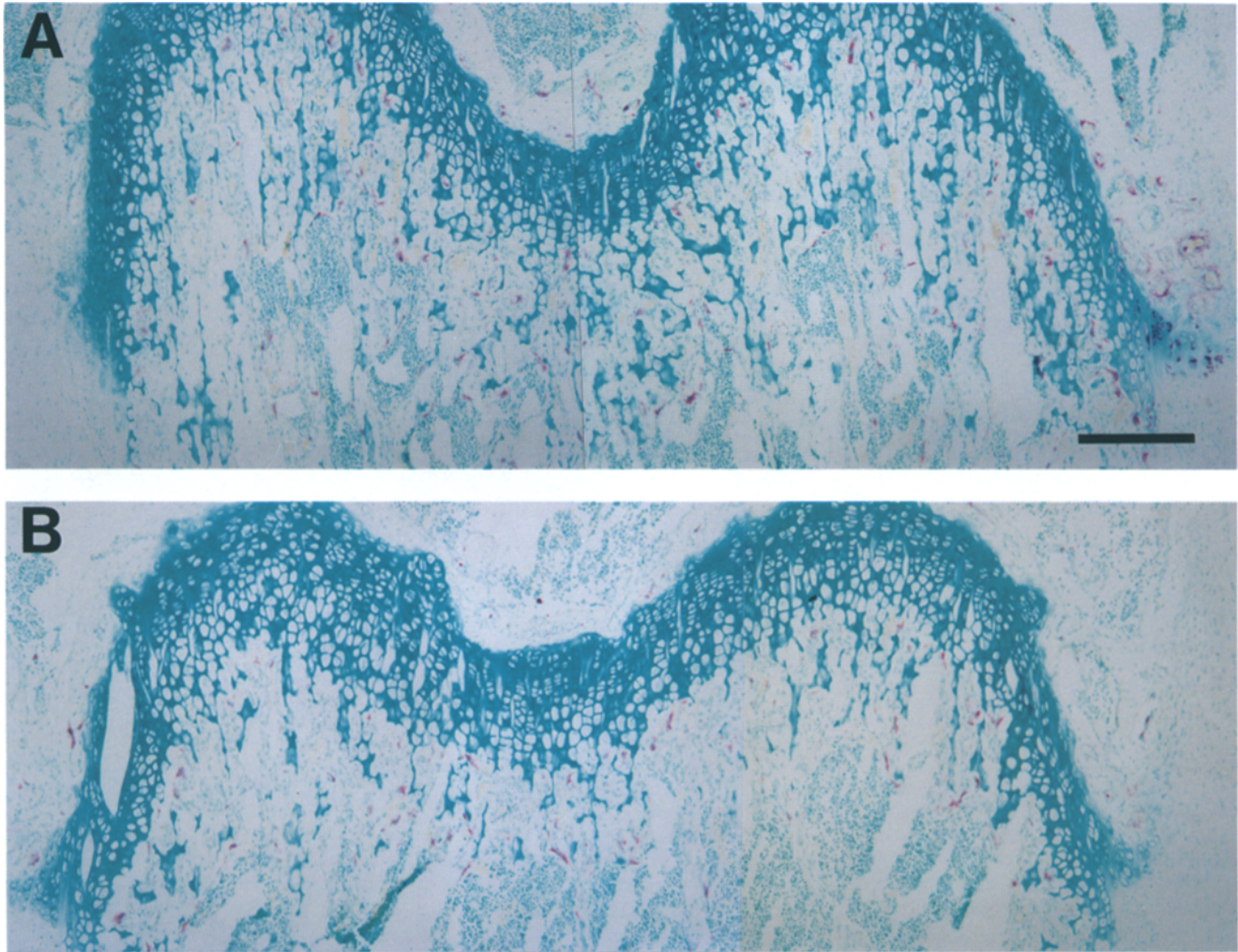
**Figure 3.** X-ray analysis of transgenic mice. (A and B) Radiographs of the pelvis, tibia, and femur of 35-d-old nontransgenic littermate (A) and D4 transgenic (B) males. Transgenic bone is generally more radiolucent, and long bones show a dramatic reduction of metaphyseal trabeculation (arrows) and thinned cortices pockmarked with multiple radiolucent defects (arrowheads). There is also a fracture of the tibia and fibula (asterisk), with a clear fracture callus formed around the tibia. Scale bar shows 0.4 mm. (C and D) Radiographs of the upper thorax of 35-d-old nontransgenic littermate (C) and D4 (D) males. Only distal vestiges of clavicles are visible in the transgenic skeleton (arrows). As with B, transgenic bone is more radiolucent than normal, and intracortical defects in the humerus are also apparent. The wild-type mouse shows an anterior arch of the atlas, a variable feature of normal mice. Scale bar shows 0.5 mm. (E–G) Radiographs of the pelvis, proximal tibia, and femur of 7-month-old nontransgenic (E) and D4 (F) and D5 transgenic (G) males. D4 bone (F) has an overall glassy appearance with a virtual absence of metaphyseal trabeculation, markedly reduced epiphyseal trabeculation, and a thinned cortex. Radiographic changes are much milder in D5 mice than in D4 mice and only a marked reduction in metaphyseal and epiphyseal trabeculation is apparent (G). Arrows show areas of metaphyseal trabeculation in the distal femur. Scale bar shows 0.4 mm.





**Figure 5.** Histological appearance of transgenic bone. (*A* and *B*) Cross sections of the tibia of 35-d-old nontransgenic littermate (*A*), and D4 transgenic (*B*) males, 3.0–3.1 mm distal to the proximal epiphyseal growth plate. In contrast to smooth endosteal and periosteal surfaces characteristic of diaphyseal bone in nontransgenic bone, D4 bone shows extensive periosteal and more pronounced endosteal resorption. In addition, cortical bone is more porous than normal (*arrows*), and the periosteal and endosteal surfaces of D4 bone are extremely fibrotic. Scale bar shows 400  $\mu\text{m}$ . (*C–E*) Cross sections of the tibia of 35-d-old nontransgenic (*C*), and D4 (*D*), and homozygous D5 (*E*) males, 3.0–3.1 mm distal to the proximal epiphyseal growth plate. Sections show equivalent areas of the tibia; *C* and *D* are high power views of areas in *A* and *B*. The dramatic increase in cellularity of D4 bone (*D*) as compared with normal bone (*C*) is evident from the increased density of osteocytes within bone matrix and the increased number of presumptive fibroblastic osteoprogenitor cells (*op*) which give rise to the osteoblasts (*ob*) lining the endosteal and periosteal surfaces. Abundant extracellular matrix contributes to the fibrotic appearance of the endosteum and periosteum. A resorption lacuna extending deep within cortical bone is marked (*closed arrow*). Areas of prior resorption refilled with new bone matrix (*light pink*) are apparent at the bone surface under the periosteum, where a scalloped cement line demarcates new from old bone (*open arrow*). Similar areas are also apparent deep within cortical bone and on nonresorbed endosteal surfaces. In contrast to D4, D5 bone (*E*) shows no detectable histological alterations from wild type. Scale bars show 100  $\mu\text{m}$ . Abbreviations: *b*, cortical bone, *m*, bone marrow, *e*, endosteum, *p*, periosteum. Decalcified, paraffin-embedded sections were stained with hematoxylin and eosin.





**Figure 6.** Histochemical identification of osteoclasts. Sections of metaphyseal trabeculation and growth plate of femurs from 35-d-old nontransgenic littermate (*A*) and D4 (*B*) females, stained for tartrate-resistant acid phosphatase activity (red), an osteoclast-specific marker. The numbers of osteoclasts in sections of control and transgenic bone are similar. Sections are counterstained with methyl green. Scale bar shows 200  $\mu\text{m}$ .

the hypocalcemia, low body weight, and soft tissue calcifications observed in the older D4 mice were secondary manifestations of prolonged bone disease.

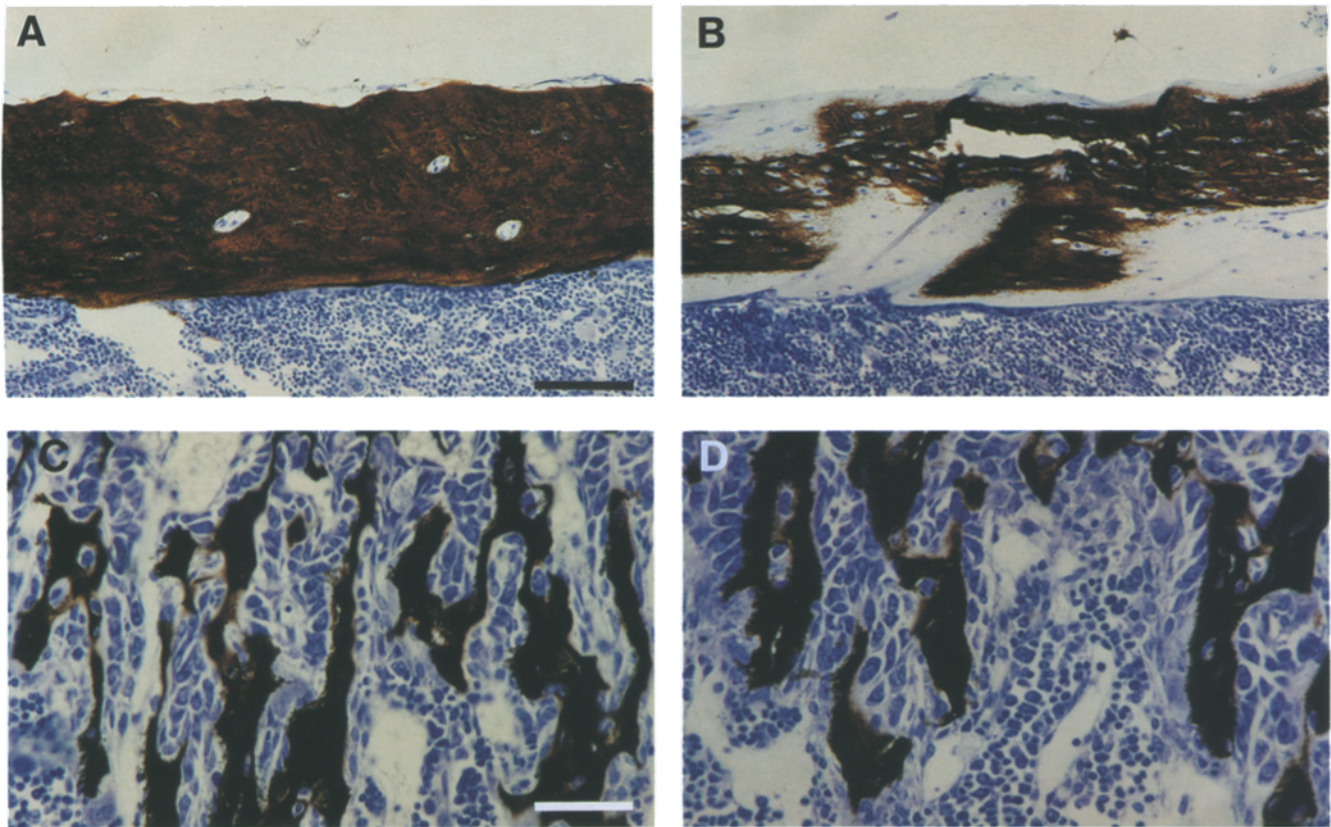
#### ***Histological Appearance of Transgenic Bone***

The progressive loss of bone mass in our overexpressing lines noted by X-ray was also apparent histologically (Fig. 4). When compared to sections of nontransgenic littermate controls at 35 d (Fig. 4 *a*), longitudinal sections through the proximal tibia of D4 mice (Fig. 4 *b*) revealed a thin cortex and a dramatic loss of trabeculation. In the metaphysis, trabecular surface density was reduced 70%, from  $13.6 (\pm 1.4) \text{ mm}^2/\text{mm}^3$  in controls to  $4.2 (\pm 2.5) \text{ mm}^2/\text{mm}^3$ . Surprisingly, the epiphyseal growth plate, which is responsible for longitudinal growth, appeared normal. Similar longitudinal sections of the tibia from 7-month-old D4 mice revealed a complete absence of metaphyseal trabeculation (with a reduction in surface density from  $5.2 (\pm 1.3) \text{ mm}^2/\text{mm}^3$  in controls to  $0 \text{ mm}^2/\text{mm}^3$ ), very little remaining epiphyseal trabeculation, and extremely thin cortical bone

(compare Fig. 4 *e* with 4 *d*). In contrast to the D4 line, hemizygous and homozygous D5 mice had only very mild alterations in phenotype at day 35 (Fig. 4 *c*, with a metaphyseal surface density similar to controls at  $15.1 (\pm 1.8) \text{ mm}^2/\text{mm}^3$ ), yet 7-month-old hemizygous mice showed a complete absence of metaphyseal trabeculation ( $0 \text{ mm}^2/\text{mm}^3$  surface density), and dramatically reduced epiphyseal trabeculation (Fig. 4 *f*).

The histological appearance of transgenic bone suggested an increase in osteoclastic resorption. The epiphyseal plates of 7-month-old D4 mice were more eroded than those of control mice (Fig. 4 *e*, arrow). At 35 d, corresponding cross-sections of the tibia at equivalent distances from the growth plate (Fig. 5) showed that cortical bone was more porous than normal and contained large resorption lacunae lined by osteoblasts (Fig. 5 *b*, arrows). Furthermore, endosteal resorption was more pronounced, and periosteal resorption, characteristic of the metaphysis, was apparent in more diaphyseal sections of transgenic bone (Fig. 5, *b* and *d*). In equivalent diaphyseal sections of normal bone, the periosteum had already assumed its





**Figure 7.** Mineralization of transgenic bone. Undecalcified sections of tibia from 35-d-old nontransgenic littermate (A and C) and D4 (B and D) females, stained by the von Kossa technique for calcium deposits (dark brown or black), and counterstained with toluidine blue. Longitudinal sections through the diaphysis reveal large unmineralized areas of cortical bone (osteoid, light blue) in transgenic bone (B), whereas wild-type bone is entirely mineralized (A). However, there is no difference in the minimal amount of osteoid present in the metaphyseal trabeculation adjacent to the growth plate (C and D). Scale bar shows 100  $\mu\text{m}$  for A and B, 50  $\mu\text{m}$  for C and D.

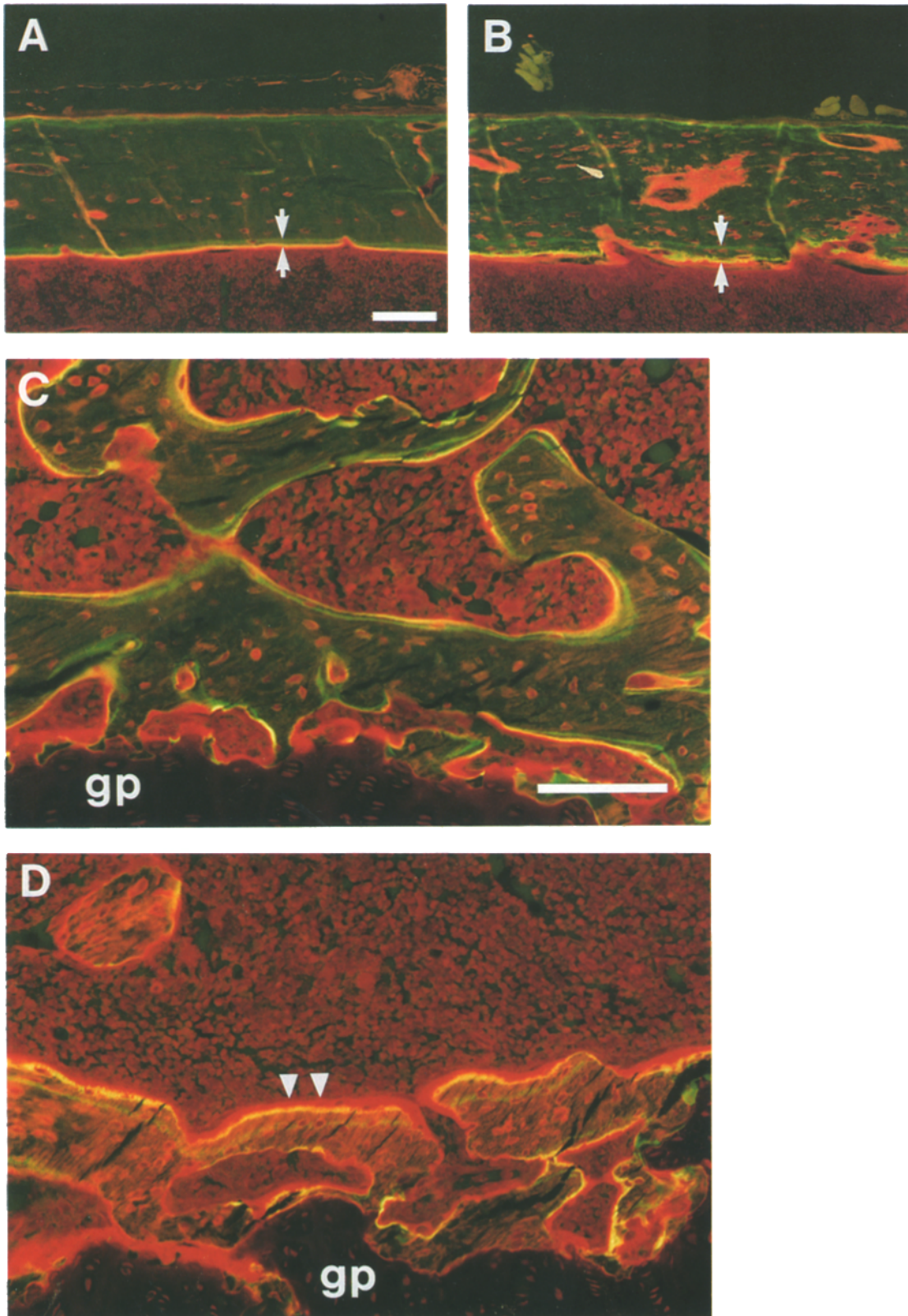
characteristically quiescent appearance (Fig. 5, a and c). Surprisingly, no striking differences in the number of osteoclasts were observed between normal and D4 mice, as assessed by the osteoclast-specific histochemical stain for tartrate-resistant acid phosphatase activity (Fig. 6).

Histological sections of 35-d-old D4 bone (Fig. 5 d) also revealed marked increases in osteocyte density (a threefold increase from 62,000 ( $\pm 14,000$ ) cells/ $\text{mm}^3$  to 197,000 ( $\pm 34,000$ ) cells/ $\text{mm}^3$ ) and presumptive osteoprogenitor cell number (a fivefold increase from 22,000 ( $\pm 8,000$ ) cells/ $\text{mm}^2$  bone surface to 120,000 ( $\pm 47,000$ ) cells/ $\text{mm}^2$ ), suggesting increased rates of osteoblastic differentiation and osteoprogenitor cell proliferation. The increased cell number along with the associated increase in extracellular matrix gave many cortical bone surfaces a distinctly fibrotic appearance. Osteoblast cell number itself remained constant at 28,000 ( $\pm 7,000$ ) cells/ $\text{mm}^2$  bone surface in transgenic bone compared to 25,000 ( $\pm 6,000$ ) cells/ $\text{mm}^2$  in

controls. On epiphyseal and trabecular surfaces, however, osteoblasts appeared more cuboidal than their nontransgenic counterparts (Fig. 2 b, open arrows), consistent with active bone matrix synthesis. Osteocyte density was also increased in bone from 7-month-old mice, however bone surfaces at this age were not fibrotic.

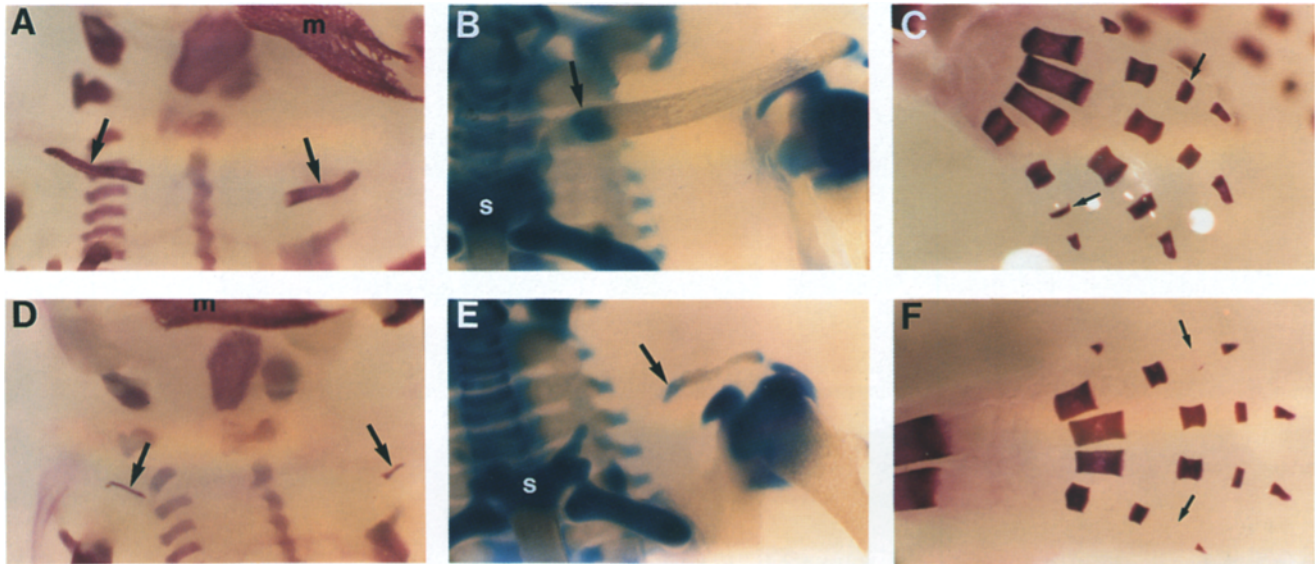
The mineralization of D4 bone was also abnormal. Bone matrix synthesized within resorption lacunae frequently appeared lightly stained in decalcified sections of cortical bone from both 35-d (Fig. 5, b and d) and 7-month-old mice. This matrix was unmineralized, as shown by undecalcified sections stained serially with hematoxylin or by the von Kossa technique, which reveals calcium deposits as dark brown or black (Fig. 7). Large pockets of unmineralized matrix extended deep within cortical bone (light blue areas in Fig. 7 b), and probably corresponded to the radiolucent intracortical defects noted by X-ray analysis. In addition, the osteoid seam, i.e., the new, as yet unmineralized

**Figure 8.** Kinetic analysis of bone turnover. Undecalcified longitudinal sections of tibia from 35-d-old nontransgenic littermate (A and C) and D4 (B and D) males viewed under UV light. Injection of calcein on day 31 and tetracycline on day 34 stains sequential sites of mineralization green and yellow, respectively. Counterstaining the sections with the Villanueva bone stain reveals unmineralized osteoid as bright red. (A and B) Longitudinal sections through cortical bone of the diaphysis. Unlike the homogenous appearance and complete mineralization of nontransgenic bone (A), transgenic bone has many pockets of unmineralized osteoid (B, bright red). These areas are not bordered by either fluorochrome label, indicating that they were not mineralizing over the course of the experiment. In



contrast, the nonresorbed endosteal surfaces of both transgenic and nontransgenic bone are mineralizing as revealed by their double labels (*arrows*). Note the increased distance between the fluorochrome labels in transgenic bone. Scale bar shows 100  $\mu\text{m}$ . (*C and D*) Higher power view of longitudinal sections through the epiphysis adjacent to the growth plate (*gp*, *deep purple*). Fluorochrome labels in the transgenic section (*D*) are diffuse and irregular as compared with their smooth appearance in nontransgenic bone (*C*). The distance between the labels as well as the width of the unmineralized osteoid layer (*bright red*, *arrowheads*) is increased over the entire length of the transgenic bone surface, indicating increased osteoblastic activity. Scale bar shows 100  $\mu\text{m}$ .





**Figure 9.** Cleared skeletal preparations of embryos and newborn mice. Nontransgenic littermate (A–C) and D4 transgenic (D–F) embryos and newborns stained with alizarin red to visualize mineralized bone or alcian blue to visualize cartilage. (A and D) Neck area of embryonic day 15.5 nontransgenic (A) and transgenic (D) embryos stained with alizarin red to reveal mineralized bone. Clavicles are fully formed in the wild-type embryo but appear as only distal rudiments in the transgenic embryo (arrows). *m*, mandible. (B and E) Neck area of 2-d-old nontransgenic (B) and transgenic (D) pups stained with alcian blue. Mineralized bone is white and cartilage is blue. No cartilage rudiment links the hypoplastic clavicle in the transgenic pup with the sternum. Arrowheads show the cartilage cap at the proximal end of the clavicle, which arises through a secondary induction event (Tran and Hall, 1989). *s*, sternum. (C and F) Forelimb paws of 2-d-old nontransgenic (C) and transgenic (F) pups stained with alizarin red. The delay in ossification of the transgenic skeleton is apparent when corresponding ossification centers are compared in transgenic and nontransgenic paws. Furthermore, other skeletal elements have started to ossify in the control paw but not in the transgenic paw (arrows).

alized layer of bone matrix deposited by osteoblasts, was wider on many nonresorbed endosteal surfaces in the epiphysis and diaphysis (see Fig. 8 *d*). In contrast, the mineralization appeared normal in metaphyseal trabecular bone, where osteoid seams were absent in both transgenic and wild-type sections (Fig. 7, *c* and *d*). Under polarized light, unmineralized matrix displayed the typical pattern of birefringence associated with lamellar collagen fiber arrangement (not shown).

The histological alterations seen in the long bones of D4 mice were also evident in cross sections of parietal bones of the skull, which develop via direct ossification rather than endochondral ossification (data not shown). In contrast to the D4 line, skeletal sections of hemizygous and homozygous D5 mice showed no microscopic histological alterations (see Fig. 5 *e* for a histological section of 35-d-old bone), except for a slightly more porous appearance of cortical bone (not shown).

### **Kinetic Analysis of Bone Turnover**

We performed a kinetic analysis of bone turnover to directly assess the synthetic activity of osteoblasts in D4 bone. Two fluorochromes that incorporate into bone matrix at sites of mineralization were injected into young mice: calcein at 31 d of age, and tetracycline 3 d later, followed by a sacrifice of the animal the next day. Undecalcified bone sections viewed under UV light visualized the incorporated fluorochromes as green and yellow fluorescent lines labeling sites of mineralization at the times of

their injection. The distance between the two lines is thus a measure of the mineral apposition rate, which correlates with the local rate of bone matrix deposition by osteoblasts (Parfitt, 1983). Counterstaining of the sections with the Villanueva bone stain simultaneously visualized unmineralized osteoid by its red fluorescence.

In long bones of 35-d-old control mice, the two fluorochrome labels formed smooth, contiguous and sharp lines (Fig. 8, *a* and *c*). In contrast, labels in transgenic bone were diffuse and irregular, and frequently interrupted by osteoid-filled resorption lacunae (Fig. 8, *b* and *d*). The distance between the two labels on many surfaces was also distinctly increased in comparison to normal bone (compare Fig. 8, *a* and *b* [arrows], and Fig. 8, *c* and *d*), corresponding to a 1.7-fold increase in the mineral apposition rate (from  $2.3 [\pm 0.3] \mu\text{m}/\text{day}$  in controls to  $4.0 [\pm 1.0] \mu\text{m}/\text{day}$ ). Almost all nonresorbed endosteal surfaces of normal bones were labeled with both fluorochromes, reflecting the overall growth of the skeleton at day 35, and the extent and distribution of these surfaces were similar in bones from D4 mice. However, the pockets of unmineralized matrix within cortical bone were not labeled with either fluorochrome, indicating a severe mineralization defect of the new bone matrix synthesized within resorption lacunae (red areas, Fig. 8 *b*). While the unmineralized osteoid seam at endosteal surfaces was wider than normal (Fig. 8 *d*, arrowheads), correlation of the osteoid seam width with the mineral apposition rate did not reveal a significant change in lag time of mineralization after osteoid deposition ( $2.2 [\pm 0.7]$  days in controls,  $2.4 [\pm 1.0]$  days in trans-

genic animals). Thus, the mineralization defect was restricted to matrix synthesized within resorption lacunae.

### ***Absence of Clavicles and Delayed Ossification***

To further evaluate the skeletal development of D4 mice, we prepared cleared skeletons at different stages of development (Fig. 9). Skeletons were stained with alizarin red to visualize mineralized bone, or alcian blue to visualize cartilage. At embryonic day 15.5, D4 clavicles were already clearly hypoplastic compared to normal embryos (compare Fig. 9, *d* and *a*, *arrows*). Since clavicle development initiates at day 14–14.5 (Tran and Hall, 1989), this difference indicates a primary defect in clavicle formation, rather than secondary resorption. As expected for the normal pattern of clavicle development through intramembranous rather than endochondral ossification (Tran and Hall, 1989), no cartilage rudiment was observed connecting the clavicle vestige with the sternum (Fig. 9, *b* and *e*).

Alizarin red staining also revealed a general delay in mineralization of the D4 skeleton, as illustrated by the ossification pattern in the hands of 2-d-old mice (Fig. 9, *c* and *f*). Whereas the lengths of corresponding digits were identical in transgenic and control mice, the mineralization of individual transgenic bones clearly lagged behind corresponding bones of normal mice. In addition, some bones of transgenic mice were not yet mineralized when compared to control counterparts (*arrows*). Importantly, the cartilaginous skeleton of transgenic embryos appeared normal, as evident from alcian blue stained preparations. With the exception of the clavicles, all other bones of D4 mice appeared normal in size and shape.

### ***Discussion***

TGF- $\beta$  is deposited in bone matrix and all three TGF- $\beta$  isoforms are expressed by bone cells *in vivo*. *In vitro* and *in vivo* studies have shown that TGF- $\beta$  can affect the activity of both osteoblasts and osteoclasts. However, the complexity of these results has made it difficult to define the normal role of TGF- $\beta$  in bone development and turnover. To provide a physiological context to study the skeletal actions of TGF- $\beta$ , we generated transgenic mice in which TGF- $\beta$ 2 is overexpressed specifically by osteoblasts. We characterized two independent lines: D5 with a 2.5-fold and D4 with a 16-fold increase in the amount of TGF- $\beta$ 2 extractable from bone matrix.

The primary phenotype of both lines was progressive, age-dependent loss of bone mass, and the severity of the phenotype correlated with the expression level of the transgene. D5 mice had no reduction of bone mass at day 35, but showed a complete loss of metaphyseal trabeculation and dramatically reduced epiphyseal trabeculation by 7 months. In contrast, bone loss in the D4 line was already striking at day 35 and extreme by 7 months. These mice also showed a defect in clavicle development, a generalized delay in the ossification of the skeleton, and a severe mineralization defect of newly synthesized bone matrix. Skeletal integrity was so compromised in the D4 line that spontaneous fractures occurred. Our inability to generate homozygous mice of this line may be related to its severe phenotype.

The progressive, age-dependent nature of the bone loss phenotype suggested a defect in bone remodeling. This was substantiated by histological and kinetic analyses of the D4 line indicating increased activities of both osteoblasts and osteoclasts in transgenic bone. The increases in mineral apposition rate and osteoid seam width on many bone surfaces indicated an increased rate of bone matrix deposition by osteoblasts. Osteocyte density and osteoprogenitor cell number were also increased, consistent with an increased proliferation of osteoprogenitor cells and an increased rate of osteoblast differentiation. Based on the osteocyte density and mineral apposition rate, we estimate that the rate of differentiation of osteoblasts into osteocytes is increased 5.6-fold in transgenic bone, from  $\sim 140$  cells/day/mm<sup>2</sup> bone surface in controls, to 790 cells/day/mm<sup>2</sup>. Since bone matrix within resorption lacunae did not mineralize over the time course of the fluorochrome-labeling experiment, we could not directly assess the rate of its production. However, it is likely that this matrix is produced at a rate equal to, if not greater than, the matrix produced on nonresorbed surfaces, because refilled lacunae usually appeared flush with the level of neighboring nonresorbed surfaces. Increased osteoclastic activity was apparent from the increased size of cortical resorption lacunae, the pronounced degree of periosteal and endosteal resorption, and the thinned epiphyseal plates of older mice, and can be inferred from the age-dependent overall decrease of bone mass in the presence of increased osteoblastic activity.

### ***The Role of TGF- $\beta$ in Bone Development and Turnover***

Our analysis indicates that overexpression of TGF- $\beta$ 2 increases the activities of osteoblasts and osteoclasts with a consequent increase in bone turnover. These results strongly suggest that TGF- $\beta$ , or specifically TGF- $\beta$ 2, is a positive regulator of bone remodeling *in vivo*. Unlike the heterogeneous results of previous *in vitro* and *in vivo* studies, our transgenic model shows that the activities of both cell types increase at steady state when TGF- $\beta$ 2 is deposited into bone matrix in analogous fashion to endogenous TGF- $\beta$ . The phenotype also shows that the increase in bone turnover results in a net imbalance between bone resorption and formation, resulting in progressive, age-dependent bone loss, and therefore suggests that TGF- $\beta$  may regulate the coordination of osteoblast and osteoclast activities. Lastly, the mineralization defect observed in these mice suggests that TGF- $\beta$ 2 may negatively regulate bone matrix mineralization *in vivo*.

Based on the documented responses of osteoblasts and osteoclasts to TGF- $\beta$  *in vivo* and *in vitro*, the increased turnover likely results from the independent actions of TGF- $\beta$  on both cell types. It is therefore unlikely that TGF- $\beta$  drives only one step of bone remodeling; instead, we speculate that TGF- $\beta$  increases the rate of remodeling by potentiating the actions of other molecular mechanisms that directly coordinate progression through the remodeling cycle. Spatial variations in endogenous TGF- $\beta$  expression levels may contribute to regional or developmental differences in remodeling or local rates of mineralization within a bone.

A priori, it was not clear that overexpression of TGF- $\beta$ 2 in osteoblasts would lead to a defect in bone turnover. Skeletal development and maintenance involve multiple levels of spatial and temporal control (Frost, 1985), and one might expect an individual growth and differentiation factor to affect several of these levels. Emerging evidence, however, suggests that the role of individual growth factors may be restricted to specific levels of skeletal organization. For example, fibroblast growth factors coordinate proportional bone growth, while the TGF- $\beta$  related factors BMP-5 and GDF-5 are required to determine the shape of several skeletal elements (for review see Erlebacher et al., 1995). Although TGF- $\beta$  expression levels during chondrogenesis could indirectly modify bone shape, TGF- $\beta$ 2 overexpression by osteoblasts does not affect bone growth, skeletal proportions, or bone morphogenesis per se (except the clavicle).

TGF- $\beta$ 2 from bone matrix was increased 2.5-fold in D5 mice. Since TGF- $\beta$ 2 is about 7.5 times less abundant than TGF- $\beta$ 1 in normal bone matrix, the progressive bone loss of these mice suggests that bone turnover may be sensitive over the long term to only minimal changes in total TGF- $\beta$  content. Additionally, TGF- $\beta$ 2 may induce different cellular or more potent responses than TGF- $\beta$ 1. This latter possibility is consistent with the higher activity of TGF- $\beta$ 2 in inducing cartilage and bone formation (Joyce et al., 1990). Such differences in the actions of TGF- $\beta$ 2 and TGF- $\beta$ 1 could correlate with differential receptor binding or expression in bone cells.

### *Regional Differences in the Bone Phenotype*

The histological changes varied depending on the area of bone. Based on the kinetic analysis and the von Kossa staining, osteoblastic activity was most perturbed in the epiphysis and diaphysis, yet appeared normal in the metaphysis. These local differences likely result from differences in the local level of expression of the TGF- $\beta$ 2 transgene. Indeed, endogenous osteocalcin expression in osteoblasts is higher in the epiphysis and diaphysis than in the metaphysis of long bones (Bronckers et al., 1985; Vermeulen et al., 1989), and expression from the transgenic promoter is accordingly higher in the diaphysis than in the metaphysis (Baker et al., 1992). As a result, increased osteoblastic expression of TGF- $\beta$ 2 in transgenic bone was more prevalent in the epiphysis than in the metaphysis. The spatial correlation between transgene expression and its phenotypic effects strongly suggests that the actions of TGF- $\beta$ 2 on osteoblasts are restricted to its site of production within bone. Furthermore, the observation that defective mineralization was restricted to the matrix within resorption lacunae supports the hypothesis that the TGF- $\beta$  within bone matrix exerts its localized effects on osteoblasts after release and activation during osteoclastic resorption (Pfeilschifter and Mundy, 1987).

In contrast to osteoblasts, the increase in osteoclastic activity in transgenic bone showed fewer regional differences. Trabecular bone mass in the metaphysis was dramatically decreased even though transgene expression was considerably less there than in the epiphysis and diaphysis. This suggests that osteoclastic activity is induced at lower local TGF- $\beta$ 2 concentrations than osteoblastic activity.

The similar numbers of osteoclasts in normal and transgenic bone further suggests that increased resorption was due to increased resorbing activity per osteoclast, a possibility consistent with the large size of resorption lacunae in transgenic bone.

### *Relationship of the Transgenic Phenotype to Human Bone Diseases*

**Cleidocranial Dysplasia.** A notable feature of D4 mice is their defect in clavicle development and general delay in ossification. Impaired clavicle development is a hallmark of the human autosomal dominant disorder cleidocranial dysplasia (Marie and Sainton, 1897; Jones, 1988) as well as the cleidocranial dysplasia (Ccd) mouse mutation (Silence et al., 1987). The Ccd phenotype also includes delayed ossification of a number of bones, and although the developmental basis of human cleidocranial dysplasia has not been well studied, delayed mineralization of the sutures and pubic bone has been noted. The severe hypoplasia or absence of clavicles in all three cases stands in contrast with the normal appearance of most other bones. However, clavicles are remarkable because they are the only bones outside the skull which develop via intramembranous ossification and not through cartilage templates (Tran and Hall, 1989). Furthermore, clavicle development strongly depends upon mechanical stimulation, which may in turn depend upon the articulation of the clavicle with the sternum (Pai, 1965; Hall, 1986). Thus, clavicle development may be uniquely sensitive to delayed ossification or to a TGF- $\beta$ 2-induced inhibition in nearby muscle development (Massagué et al., 1986; Olson et al., 1986). The increased osteoblastic expression of TGF- $\beta$ 2 in D4 transgenic mice may provide a starting point to study the developmental abnormalities associated with human cleidocranial dysplasia and the mouse Ccd phenotype.

**Hyperparathyroidism.** The phenotype of the high TGF- $\beta$ 2 expressing D4 line showed a remarkable similarity to the skeletal abnormalities associated with hyperparathyroidism, a disease resulting from increased circulating levels of parathyroid hormone (PTH) (Malluche and Faugere, 1990; Kronenberg, 1993). Common features include generalized osteopenia, increased density of osteocytes, increased osteoclastic resorption, and high turnover kinetics. Increased amounts of unmineralized osteoid apparent in our transgenic mice are also commonly seen in hyperparathyroidism, especially when associated with renal failure (renal osteodystrophy), and fibrotic bone surfaces reflect a severe hyperparathyroid state termed osteitis fibrosa.

PTH is thought to maintain systemic calcium homeostasis in part by regulating osteoclastic bone resorption via an indirect mechanism involving osteoblasts (Rodan and Martin, 1981; McSheehy and Chambers, 1986; Kronenberg, 1993). How increased PTH levels lead to the skeletal defects in hyperparathyroidism is unknown. The similarity of the D4 phenotype with hyperparathyroidism may link the local effects of TGF- $\beta$ 2 to the systemic actions of PTH. The osteoblast-dependent PTH stimulation of osteoclastic activity in vitro is simulated by TGF- $\beta$  (Hattersley and Chambers, 1991), and PTH induces TGF- $\beta$  expression and activation by osteoblasts in vitro (Oursler et al., 1991; Yee et al., 1993) and TGF- $\beta$  expression in bone in vivo (Pfeil-

schifter et al., 1995). In addition, PTH alters the response of osteoblasts to TGF- $\beta$  in culture, perhaps by altering the TGF- $\beta$  receptor expression pattern of these cells (Centrella et al., 1988). Conversely, TGF- $\beta$  upregulates the expression of PTH receptors in osteoblasts (Schneider et al., 1992; Seitz et al., 1992), raising the possibility that increased TGF- $\beta$  expression mimics the histological phenotype of hyperparathyroidism by sensitizing osteoblasts to physiological levels of PTH. It is highly unlikely that the severe D4 phenotype resulted from increased levels of PTH or PTH-related peptide, since transgenic mice showed no evidence of hypercalcemia or hypophosphatemia (Kronenberg, 1993; Strewler and Nissenson, 1993).

**Osteoporosis.** Finally, the mild phenotype of the D5 line, with its progressive, age-dependent bone loss with little or no alteration in the microscopic appearance of bone, closely resembles osteoporosis, a prevalent disease thought to result from an imbalance between osteoclastic and osteoblastic activities during bone remodeling (for review see Manolagas and Jilka, 1995). In addition, bone from 7-month-old D4 mice were extremely osteoporotic, aside from the increased osteocyte density and mineralization defect, which are, strictly speaking, not features of osteoporotic bone. Furthermore, a hallmark of osteoporosis is the increased propensity towards fracturing. Spontaneous fractures were indeed commonly seen in D4 mice, and we infer that D5 mice may therefore be more prone to fractures as well.

Despite the association of osteoporosis with changes in the hormonal regulation of bone turnover, such as reduced estrogen in the postmenopausal state, the local mediators which alter bone remodeling and lead to the imbalance between osteoclastic and osteoblastic activities at the histological level remain largely unknown. Considering the epidemiological and histological heterogeneity of osteoporosis (Meunier et al., 1980; Parfitt et al., 1980; Whyte et al., 1982), and the multiplicity of cytokines and growth factors that affect osteoblasts and osteoclast function in vitro (Mundy, 1993), it is likely that several factors influence bone turnover and contribute to the pathogenesis of osteoporosis. Thus, transgenic overexpression of interleukin-4 by T cells leads to an osteoporotic phenotype with decreased osteoblastic function, suggesting that this factor could play a role in low-turnover osteoporosis (Lewis et al., 1993). Furthermore, mice-deficient mice in interleukin-6 (IL-6) expression have revealed a requirement for this cytokine in the bone loss that follows ovariectomy, which resembles postmenopausal osteoporosis (Poli et al., 1994). Interestingly, IL-6-deficient mice have an increased rate of bone turnover but no discernible bone loss before ovariectomy.

Our transgenic mice show that increased osteoblastic expression of TGF- $\beta$ 2, a normal product of osteoblasts in vivo, causes both an increase in bone remodeling and an imbalance in osteoclastic and osteoblastic activity leading to progressive bone loss, a phenotype similar to high-turnover osteoporosis. A potential link between postmenopausal osteoporosis and TGF- $\beta$  has previously been made with the finding that ovariectomy in rats leads to a 40% specific reduction in TGF- $\beta$  extractable from bone matrix (Finkelman et al., 1992). However, it is unclear how this reduction is causally related to the high turnover state fol-

lowing ovariectomy (Wronski et al., 1985, 1986), since we see a similar reduction in TGF- $\beta$ 1 content of bone matrix in our mice overexpressing a high level of TGF- $\beta$ 2. The remarkable association of an osteoporosis-like phenotype with only a mild, i.e., 2.5-fold increase in TGF- $\beta$ 2 expression by osteoblasts that only minimally affects the total TGF- $\beta$  content of bone, suggests that small alterations in the expression of TGF- $\beta$  in bone, or in the responsiveness of bone cells to TGF- $\beta$ , could contribute to the remodeling defects underlying osteoporosis and other metabolic bone diseases.

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