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Impaired Bone Formation in Transgenic Mice Resulting from Altered Integrin Function in Osteoblasts

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To determine the role of integrins in mature osteoblasts *in vivo*, we expressed in transgenic mice a dominant-negative integrin subunit (β1-DN) consisting of the β1 subunit cytoplasmic and transmembrane domains, driven by the osteoblast-specific osteocalcin promoter. Immature osteoblasts isolated from transgenic animals differentiated normally *in vitro* until the osteocalcin promoter became active; thereafter they detached from the substratum, suggesting that β1-DN was impairing adhesion in mature osteoblasts. Transgenic animals had reduced bone mass, with increased cortical porosity in long bones and thinner flat bones in the skull. At 35 days, the rate of bone formation was reduced in cortical bone, and the parietal bones were 45% thinner than in wild-type animals. Active osteoblasts were less polar and had larger areas of cytoplasm with intracellular stores of matrix molecules. Osteocyte lacunae appeared normal around the cell body but did not have normal canilicular structures. At 90 days, the parietal bone of transgenic males was of normal width, suggesting that the original defect in matrix deposition had been repaired or compensated for. In contrast, transgenic females still had decreased bone mass in the parietal bone at 90 days. The decreased bone mass in TG females was accompanied by increased staining for osteoclast activity, suggesting that there was a sex-specific defect in mature animals.

Key Words: **osteoblast; transgenic mice; integrins; dominant negative; bone development.**

INTRODUCTION

Bone is a highly dynamic tissue that is continually repaired and remodeled in response to physiological and environmental stimuli, such as systemic hormones and mechanical strain. The formation and homeostasis of all tissues require extensive interactions between specialized cells and the extracellular matrix (ECM)[1] (Damsky *et al.*, 1997). Bone is a model tissue in which to study these types of interactions, because it has a large and extensive ECM that is secreted and organized by a single cell type, the osteoblast. These cells, in turn, are a model for studying cell–ECM interactions, because they have multiple complex interactions with ECM that change as they differentiate from mature osteoblasts into osteocytes. It is not well understood, however, how osteoblasts coordinate bone formation or respond to external stimuli (e.g., mechanical forces, systemic hormones) or how cell surface receptors are involved in these processes.

Integrins are a major family of ECM receptors that transduce signals from the environment to the cell interior. Signals from integrin ECM receptors can synergize with signals from growth factor receptors to regulate downstream cellular functions such as cytoskeletal organization, gene expression, and apoptosis (Damsky and Werb, 1992; Miyamoto *et al.*, 1996; Giancotti and Rouslahti, 1999). Osteoblasts have a rich repertoire of integrin receptors (Clover *et al.*, 1992; Gohel *et al.*, 1995; Grzesik and Robey, 1994; Hughes *et al.*, 1993; Moursi *et al.*, 1997; Saito *et al.*, 1994). Using osteoblast cell culture systems (Bellows *et al.*, 1986), we and others have shown that integrin interactions with fibronectin (FN) and collagen type I (Col I) are critical

[1] Abbreviations used: Col I, collagen type I; ECM, extracellular matrix, FN, fibronectin; LN, laminin; MSC, marrow stromal cells; TG, transgenic; WT, wild type.

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for osteoblast differentiation (Gronowicz and Derome, 1994; Moursi *et al.*, 1996, 1997) and survival (Globus *et al.*, 1998). Although primary cell systems are useful, they do not represent a physiological environment, because they do not accurately reproduce the three-dimensional structure of bone matrix, and they are not subject to multiple factors present *in vivo* (i.e., hormones, mechanical loading). To better understand how integrin functions regulate osteoblast behavior *in vivo*, it is necessary to establish appropriate genetic models.

Mutations in bone matrix molecules often result in striking skeletal phenotypes, some of which are analogous to human diseases such as osteogenesis imperfecta, osteoarthritis, and chondrodysplasia (Faessler *et al.*, 1996). However, there are no known genetic models of defective integrin function in bone formation, and therefore the potential contribution of integrin receptors to skeletal diseases is still unclear. Null mutations of individual integrin subunit genes have not provided suitable models to study integrin involvement in bone formation, either because they result in embryonic lethality or because they have no detectable bone phenotype (Brakebusch *et al.*, 1997; Yang *et al.*, 1993). Integrins of the β1 family, for example, are essential for normal development, and inactivation of the β1 gene results in early embryonic death (Faessler and Meyer, 1995; Stephens *et al.*, 1995).

To overcome these limitations, we targeted the expression of a dominant-negative β1 integrin subunit (β1-DN) to mature osteoblasts *in vivo*. Expression of single-subunit chimeras containing β integrin intracellular and transmembrane domains but lacking the extracellular domain can function in a dominant-negative fashion to inhibit endogenous integrin function in cell adhesion, spreading, migration, and matrix assembly (Chen *et al.*, 1994; Faraldo *et al.*, 1998; LaFlamme *et al.*, 1994; Lukashev *et al.*, 1994; Smilenov *et al.*, 1994). Expression of β1-DN was controlled by the osteocalcin promoter, which is expressed only in mature osteoblasts and osteocytes (Baker *et al.*, 1992; Mikuni-Takagaki *et al.*, 1995) and has been successfully used to target transgene expression to skeletal structures in transgenic (TG) mice (Baker *et al.*, 1992; Erlebacher and Derynck, 1996; Frenkel *et al.*, 1997). TG animals, which thus had altered integrin function in mature osteoblasts and osteocytes, had a primary defect characterized by reduced bone mass in cortical and flat bones. The rate of cortical bone formation was also reduced in TG animals, and the bone that was formed appeared more disorganized. Mature transgenic females had a secondary defect characterized by less bone mass in flat bones accompanied by increased staining for osteoclasts.

MATERIALS AND METHODS

Plasmid Construction

HA-β1-DN contained 118 nucleotides of the preprolactin signal sequence, including 18 nucleotides before the first ATG and 10 nucleotides downstream of the signal peptidase cleavage site, cloned in frame with the coding sequence for 9 amino acids of an immunogenic peptide of hemagglutinin (HA-tag), YPYDVPDYA (Hamel *et al.*, 1992), followed by the coding sequence for the C-terminal 83 amino acids of human β1 (including the cytoplasmic tail and transmembrane domains). The transgene construct contained 1.8 kb of the rat osteocalcin promoter, 640 nucleotides of the rabbit β-globin intron, HA-tagged β1-DN, and 627 nucleotides from the human growth hormone poly(A) tail cloned into Bluescript and flanked by two unique restriction sites (*Spe* and *Not*I) (Fig. 1).

Transgenic Mice

DBA/2 × C57BL/6 F1 (B6D2 F1) mice were used to generate TG mice using standard techniques (Hogan *et al.*, 1986). We injected the transgene construct as a 2.4-kb fragment excised from pOC-HA-β1 with *Spe* and *Not*I and purified by Qiex Gel Extraction. TG mice were identified by Southern blot analysis of the tail DNA (Laird *et al.*, 1991), using the β-globin intron fragment as a hybridization probe. TG lines were established and maintained on a B6D2 F1 background. Subsequent generations of TG mice were identified by PCR analysis or by dot blots, using the β-globin intron as a probe. Heterozygous TG mice were bred to generate homozygous TG and wild-type (WT) mating pairs. These were subsequently bred for phenotype analysis.

RNA Analysis

RNA analysis was done as described (Erlebacher and Derynck, 1996), except that the 3′ primer used was specific for the HA-β1 gene.

Histomorphometry and Histological Analysis

Sample preparation. For kinetic analysis of bone formation, mice were injected subcutaneously with the fluorochromes calcein (10 mg calcein/kg mouse) and tetracycline (25 mg tetracycline/kg mouse). Mice that were sacrificed on day 35 were injected with calcein on day 30 and tetracycline on day 34. Dissected femur and calvarial bones were immediately fixed in 70% EtOH and stored at 4°C. All samples were postfixed for 48 h in 10% neutral-buffered formalin and equilibrated into PBS for 48 h. For plastic-embedded sections, the samples were transferred to Villanueva bone stain (osteochrome stain; Polysciences, Inc., Niles, IL) for 48 h, dehydrated in graded ethanols, and embedded in methylmethacrylate. For paraffin-embedded sections, the samples were decalcified for 2 weeks in a PBS solution containing 10% EDTA, equilibrated back into PBS for 48 h, dehydrated with EtOH, and then embedded. All immunocytochemistry was done using paraffin sections that were postfixed with MeOH. Paraffin sections were 5 μm and plastic sections 4.5 μm in thickness. Histomorphometric measurements were made using the Osteomeasure program from Osteometrics (Atlanta, GA).

Cortical bone measurements. Cortical bone measurements were made on cross sections of paraffin- or methylmethacrylate-embedded femurs taken approximately 4 mm from the growth plate. Porosity of the cortical bone was measured using Osteomeasure software by tracing the perimeter of the cortical bone within one field of a cross section viewed through the 20× objective. Porous areas within the traced perimeter were measured, and percentage porosity was determined as % porous = porous area/total area × 100. Two fields from a single section were measured

for each animal, and the average porosity of these fields was reported. Quantification was based on the analysis of cortical bone from 11 TG and 6 WT animals. Measurements of trabecular area and thickness were made on one section per animal viewed through a 20× objective; $n = 9$ for WT and 10 for TG animals. The bone formation rate ($\mu m^3/\mu m^2$/day) was determined by tracing fluorochrome labels on the endosteal surface of cortical bone. Measurements were made of three separate 20× fields on a single section. One section was analyzed per animal; $n = 4$ for both WT and TG animals.

Parietal bone measurements. Embedded parietal bones were sectioned parallel to the occipital suture at a distance of ~1 mm from the suture. Sections were viewed through the 20× objective, and width and lamellar organization were measured on the center field in each section. The width of the parietal bone was determined by tracing the perimeter of the bone within the field and calculating the average width over the field. One section was analyzed per animal. Measurements of width at 35 days were made by analyzing parietal bones from 10 TG females, 4 TG males, 7 WT females, and 5 WT males and at 90 days from 4 WT females, 5 TG females, 6 WT males, and 9 TG males. To quantify the percentage of lamellar bone, the sections were viewed through polarized light to visualize the birefringence pattern of the collagen matrix, and the area of lamellar bone was determined as % lamellar = lamellar area/total bone area × 100. Quantification was based on the analysis of 35-day parietal bones from 10 WT and 11 TG animals.

Mouse Marrow Stromal Cell Cultures

Bone marrow was extruded from the femurs and tibias of 35-day-old mice and plated in α-MEM containing 15% FCS (Leboy *et al.*, 1991). Bone marrow from the femurs and tibias of one animal was pooled and plated into two six-well tissue culture wells. The cells were incubated at 37°C in 5% CO_2, and the medium was replaced every 2–3 days until the cultures were nearly confluent. At this time the cells were trypsinized and replated at a density of 1.25×10^4 cells/cm^2/0.5 ml medium. The next day (day 1) the medium was replaced with inducing medium (α-MEM containing 15% FCS, 200 μM ascorbate phosphate, β-glycerol phosphate, and 100 ng/ml bone morphogenic protein-2), which was subsequently replaced every 3 days until the cultures were used for analysis.

Adenovirus Production

Adenoviruses were produced as previously described (Hardy *et al.*, 1997). To make an adenovirus expressing green fluorescent protein (GFP) a 0.8-kb DNA fragment containing the GFP gene was removed from pEGFP-1 (Clontech, Palo Alto, CA) and cloned into the pADlox vector for production of recombinant adenovirus. To make an adenovirus expressing GFP from the osteocalcin promoter, the CMV promoter in the pADlox plasmid was replaced with a 1.8-kb osteocalcin promoter.

Whole-Mount TRAP Staining

For whole-mount staining of osteoclasts with tartrate-resistant alkaline phosphatase (TRAP), calvaria were prepared using a leukocyte acid phosphatase kit (Sigma Chemical Co., St. Louis, MO) with the coupling dye replaced by Fast red-violet LB (Sigma) (Erlebacher *et al.*, 1998). Calvaria were fixed for 10 min in 2% paraformaldehyde and 0.8% glutaraldehyde, followed by overnight fixation in 70% EtOH. Calvaria were postfixed in MeOH for 1 h to inhibit non-cell-associated TRAP staining. Similar results were observed when alternative protocols for TRAP staining were used.

X-ray Analysis

Dissected calvaria were fixed in 70% EtOH at 4°C and radiographs of the fixed calvaria were taken on a Faxitron Model 43805 N (Hewlett–Packard, Palo Alto, CA), using Kodak X-OMAT TL film. All X-rays used for quantification or for comparison were taken simultaneously, and exposure was for 30 s at 35 kVp.

Scanning Electron Microscopy

Bone samples were prepared for scanning electron microscopy analysis as previously described (Boyde and Jones, 1996). Organic matrix components were removed from the samples by treatment with a solution of NaOCl with 7% available chlorine content for approximately 1 week, and the samples were washed extensively with ddH_2O, before being prepared for analysis.

RESULTS

Generation of Transgenic Mice

A 2.4-kb transgenic construct (Fig. 1A) that included a 1.8-kb segment of the rat osteocalcin promoter (Baker *et al.*, 1992), a globin intron, the HA-β1 gene, and a human polyadenylation site was used to generate TG mice (Erlebacher and Derynck, 1996). Five TG founder lines were identified by Southern blotting (Fig. 1B) and PCR analysis of genomic DNA (data not shown). The founder lines were tested for expression of transgene mRNA by reverse transcription-PCR (Fig. 1B). Two lines were followed for more extensive histological analysis. The phenotype was more severe in animals homozygous for the transgene, suggesting that the level of expression of the transgene affected the severity of the phenotype (data not shown).

Immunostaining using an anti-HA antibody (Fig. 2A) showed that the β1-DN construct was expressed by osteoblasts (arrows) and osteocytes (arrowheads). Endogenous β1 was localized by immunocytochemistry to osteoblasts on the bone surface and osteocytes within the bone matrix in a similar manner in sections from WT and TG animals (Figs. 2B and 2D). Hoechst staining was used to visualize nuclei and showed that osteoblasts expressing the β1-DN appeared healthy and nonapoptotic (Fig. 2C). There did not appear to be any mineralized surfaces denuded of cells, suggesting that mature osteoblasts had not detached from the bone surface.

The Transgene Is Appropriately Expressed by Mature Osteoblasts in Vitro, and Transgene Expression Results in Defective Adhesion

Primary stromal cells cultured from bone marrow differentiate into osteoblasts over a 2-week period when grown in the presence of bone morphogenic protein-2. Cells isolated from both WT and TG bone marrow stroma grew and

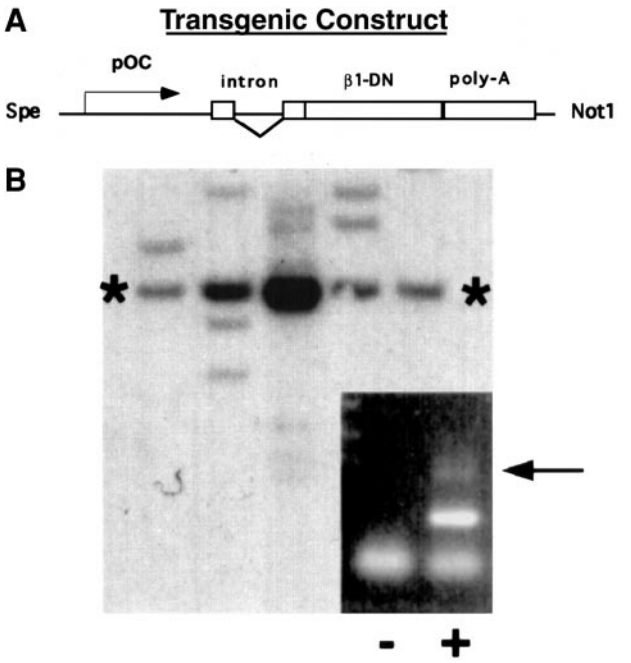

FIG. 1. (A) The transgenic construct contains a 1.8-kb fragment of the osteocalcin promoter followed by a globin intron, the β1-DN gene, and a poly(A) tail. The 2.4-kb construct is flanked by unique restriction sites for *Spe* and *Not*I. (B) Five founder lines were established and the transgene is expressed in bone. Five founder lines were identified by Southern analysis. The star denotes a common restriction fragment within the β1-DN gene; the band beneath the bar is due to nonspecific background. The inset shows the results of reverse transcriptase-PCR on total RNA extracted from TG animals in the absence (−) or presence (+) of primers for the β1-DN gene. The band of the appropriate size is indicated by an arrow.

differentiated normally over this time period. The majority of cultured cells from both WT and TG animals were osteoblastic and after 9 days in culture stained positively for the early osteoblast differentiation marker, alkaline phosphatase (data not shown). To determine whether activity from the osteocalcin promoter was high enough to use marrow stromal cells (MSC) as a model to assess transgene expression and function *in vitro,* we tested for the activity of the osteocalcin promoter using GFP as a reporter gene. WT MSC were infected with a recombinant adenovirus expressing GFP from the osteocalcin promoter or, as a control, GFP from the CMV promoter. Our results indicate that the CMV promoter was equally active in nodular and internodular cells (Fig. 3A) in mature cultures (13 days after plating). In contrast, the osteocalcin promoter was highly active in osteoblasts within bone-like multilayered nodules, as assessed by GFP expression (Fig. 3B), and was much less active in osteoblasts that were in the internodular areas. These results show that the osteocalcin promoter is highly active in MSC *in vitro,* that osteocalcin is preferentially expressed in mature osteoblasts within bone-like nodules, and that MSC cultures should provide some insight into the expression and mechanism of function of the β1-DN gene.

In TG cultures, the β1-DN protein was detected by IF using antibodies against the HA-tag (Fig. 3C). Double-fluorescence microscopy showed that at day 9, the cells that contained the highest level of osteocalcin promoter activity, as determined by GFP expression (Fig. 3D), also had the brightest staining for the transgene product (compare Figs. 3C and 3D). These results suggest that the β1-DN gene was appropriately regulated by the osteocalcin promoter *in vitro.* Osteoblasts expressing high levels of the transgene appeared healthy and not apoptotic (Fig. 3E) and for the first several days after the initiation of osteocalcin promoter activity remained spread and attached to the substratum (Fig. 3D).

Several days after the initiation of osteocalcin promoter activity, WT cultures remained in multilayered sheets attached to the culture dish (Fig. 4A). In contrast, most of the cells in TG cultures, including all cells with strong activity from the osteocalcin promoter, detached from the substratum as a sheet and contracted into three-dimensional structures (Fig. 4B). After several additional days in culture, individual cells and small clusters of TG cells detached from the contracted three-dimensional structure (Fig. 4C). When medium conditioned by TG cultures was transferred to WT cultures, there was no effect on cell attachment or matrix organization (data not shown), suggesting that cell matrix contraction and cell detachment were intrinsic behaviors of the TG cells and were not caused by secreted factors. Taken together these data suggest that β1-DN is highly expressed on the cell surface of mature osteoblasts and that *in vitro,* the expression levels are high enough to perturb endogenous integrin function and interfere with cell adhesion.

TG Cortical Bone Is Porous and Has a Decreased Rate of Bone Formation

Osteocalcin is a late marker for mature osteoblasts. In mice, the osteocalcin promoter is not highly expressed until 1–2 days after birth, a time after which the initial skeletal template has been patterned and formed but before the collagen matrix has been extensively mineralized. Accordingly, no change in the size or pattern of the skeleton was observed in TG animals compared with WT animals (data not shown).

To assess the integrity and structure of bone matrix, TG and WT bones were analyzed histologically. The cortical bone from 12-day WT animals had a smooth surface when viewed in cross section (Fig. 5A(a)). In addition, the bone on the interior was solid and contiguous and was not disrupted by large porous areas (Fig. 5A(a)). In contrast, the cortical bone of TG animals was highly porous and noncontiguous on the interior and had an irregular noncylindrical shape on the surface (Fig. 5A(b)). The percentage of bone that was

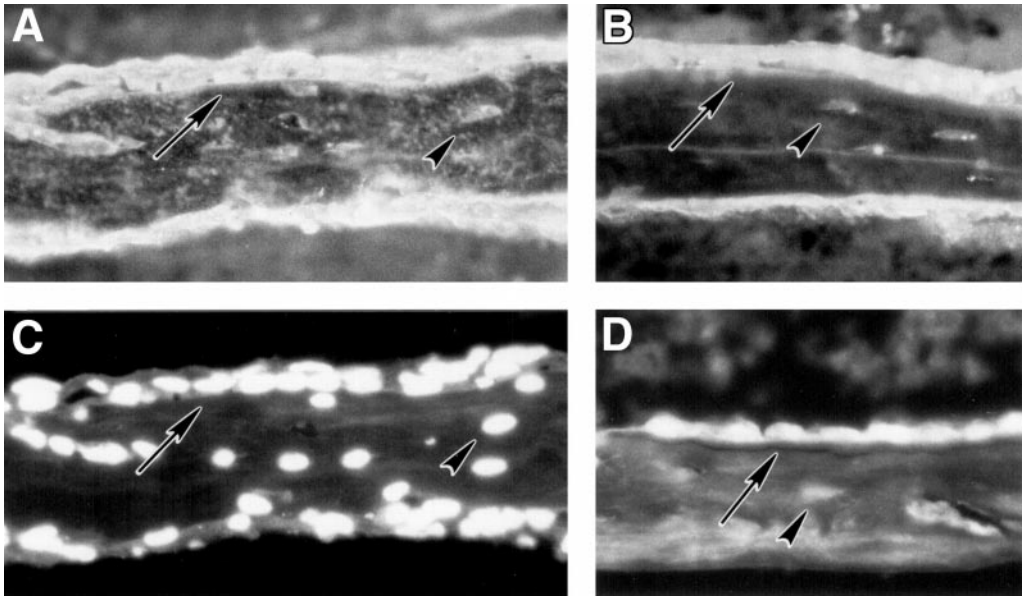

FIG. 2. β1-DN is expressed by TG osteoblasts and osteocytes. Cross sections through the parietal bones of 35-day TG (A, B, and C) and WT (D) animals stained by IF with antibody against the HA-tag in β1-DN (A) or the extracellular domain of β1 (B and D).(C) Fluorescence microscopy showing Hoechst nuclear staining of the TG section shown in (A). Arrows, osteoblasts; arrowheads, osteocytes; all photomicrographs are shown at 200× magnification.

porous rather than solid bone matrix was analyzed quantitatively (Fig. 5B). The cortical bone of TG animals was 160% more porous than WT bone (Fig. 5B), while the average thickness of the cortical bone in TG animals was similar to that of WT (data not shown). These data indicate that TG bone has significantly less bone mass than WT bone. In contrast, trabecular bone mass was normal in TG animals as assessed by measuring bone volume and trabecular thickness (data not shown).

The rate of bone formation was measured on the endosteal surface of cortical bone at 35 days by tracing fluorochrome labels. In WT animals the bone formation rate was 8.4 $\mu m^3/\mu m^2$/day, whereas in TG animals it was 3.25 $\mu m^3/\mu m^2$/day. Thus the rate of bone formation in TG cortical bone was approximately 40% that of WT animals ($P < 0.05$, t test), and this decreased bone formation rate may account for the decreased bone mass observed in TG animals.

TG Parietal Bone Is Thinner and More Disorganized

The flat bones of the skull are formed by intramembranous rather than by endochondral bone formation. Thus, unlike cortical bone they are formed *de novo* and not within a cartilage template. To determine the effects of β1-DN expression on intramembranous bone formation, we analyzed histologically parietal bones, which are the largest flat bones of the skull. Cross sections of parietal bones from WT and TG animals were analyzed for thickness and for the organization of the bone matrix.

The parietal bones of TG animals were thinner than those of WT (Fig. 6) and therefore had less bone mass. The parietal bones of 35-day TG females were 30% thinner than those from 35-day WT females (Fig. 6A, compare (b) and (d)). Interestingly, the difference was most striking between TG males and WT males (Fig. 6A, compare (a) and (c)). The parietal bones of 35-day TG males were 45% thinner than those of WT (Fig. 6B). Females normally have thinner flat bones than their male counterparts, so the net result of these differences was that in the TG animals the parietal bones were effectively the same thickness in males and females (Fig. 6A, compare (c) and (d)). These observations suggest that in the TG animals, bone formation was limited by integrin function during the time of rapid bone formation that occurs during growth.

At 35 days, the skeleton is mature in WT animals, and the period of rapid skeletal growth has just concluded; thus the majority of bone matrix present should be in a mature form. Accordingly, in 35-day WT animals 66% of the parietal bone was arranged in an ordered lamellar structure that was easily visualized through polarized light (Fig. 7). However, in TG animals of the same age, only 25% of the parietal bone had a similar structure (Fig. 7). The remainder of the bone matrix appeared disorganized and resembled less-mature woven bone, suggesting that in TG animals ECM maturation is either defective or delayed.

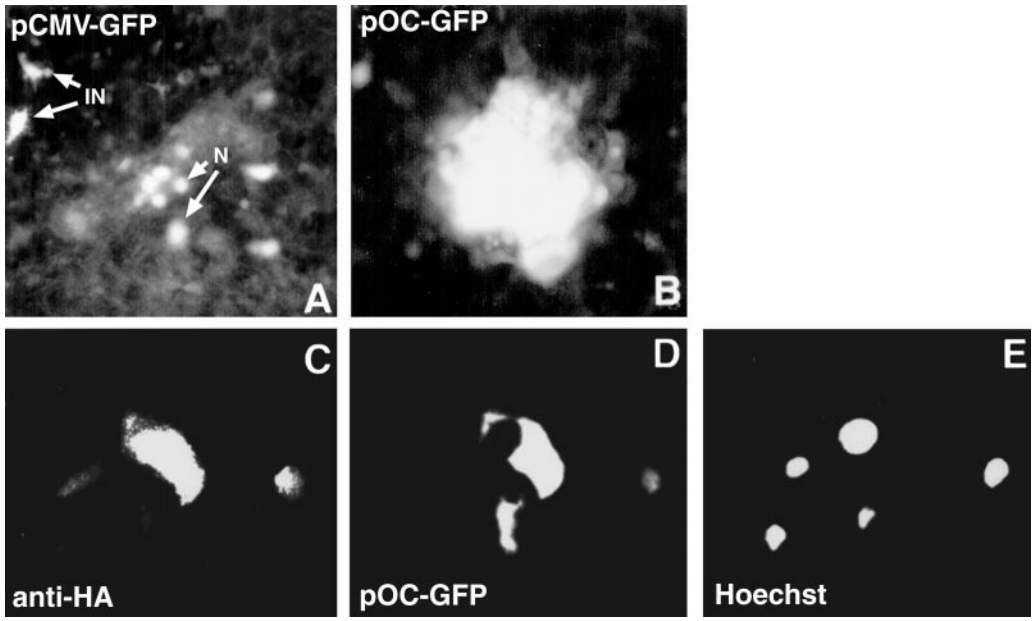

FIG. 3. The osteocalcin promoter is active in mature bone-like nodules, and β1-DN is expressed by primary osteoblasts *in vitro*. 13-day MSC were previously infected with a recombinant adenovirus that expresses GFP from the CMV promoter (A) or from the osteocalcin promoter (B). Arrows in (A) indicate GFP-expressing cells in nodular (N) and internodular (IN) areas of the culture. Day 9 cultures were previously infected with a recombinant adenovirus that expresses GFP from the osteocalcin promoter (D). GFP-expressing cultures were stained with Hoechst (E) and then fixed and stained by IF with antibodies against the HA tag (C). Triple IF of the same field of cells shows that cells expressing high levels of GFP from the osteocalcin promoter (D) also express high levels of β1-DN expressed on the cell surface (C). Cells expressing high levels of β1-DN have intact nuclei (E) and are not apoptotic. The photomicrographs in (A) and (B) are shown at 100× magnification. The photomicrographs in (C–E) are shown at 320× magnification.

In TG Animals ECM Organization Is Abnormal

Most WT osteoblasts were polarized, with the nucleus positioned at the end of the cell away from the bone surface and the cytoplasm and secretory organelles located close to the cell surface in contact with the bone matrix. In TG animals, mature osteoblasts often lacked this distinctive intracellular arrangement and resembled nonpolarized cells (Figs. 8A and 8B). TG osteoblasts also had larger intracellular regions that stained strongly for type 1 collagen (Figs. 8C and 8D) and laminin (LN) (data not shown). Quantitative

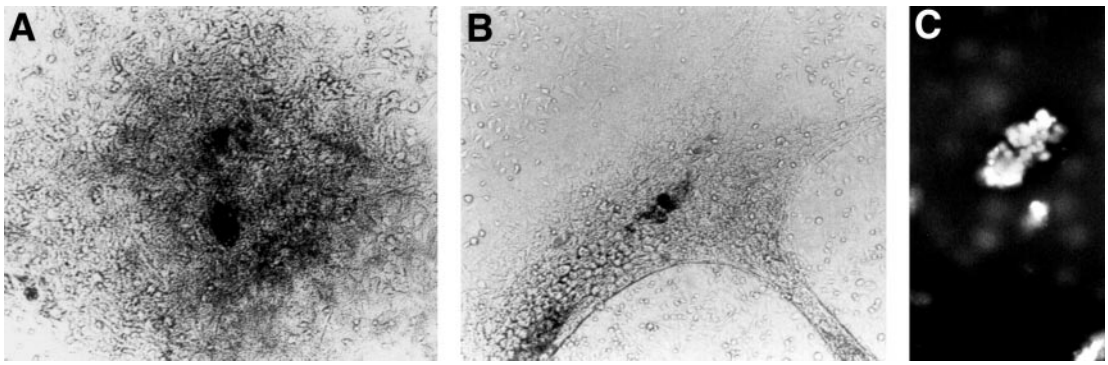

FIG. 4. TG osteoblast cultures have an adhesion defect *in vitro*. MSC cultures derived from WT (A) and TG (B) animals are shown at 16 days after plating. (C) A cluster of detaching TG MSC previously infected with pOC-GFP is observed by fluorescence microscopy. The photomicrographs in (A) and (B) are shown at 100× magnification; (C) is shown at 320× magnification.

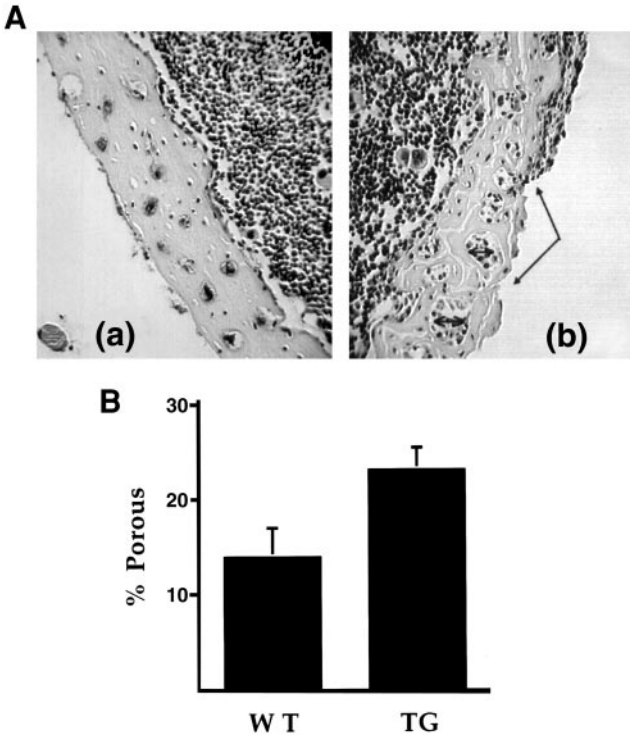

FIG. 5. In TG animals cortical bone is highly porous and irregular. (A) Cross sections of paraffin-embedded cortical bones from 12-day WT (a) and TG (b) animals. The inner and outer surfaces of cortical bone from TG animals (b) were highly irregular (arrows), compared with cortical bone from WT animals (a), and the interior of the cortex was highly porous. (B) Quantitative measurements of porosity in cortical bone. The bars denote SEM. The differences between WT ($n = 6$) and TG ($n = 11$) are statistically significant ($P < 0.03$, t test).

measurements were made of the cell area that stained positively for Col I. On average, TG osteoblasts had 40% more cytoplasm containing strong staining for Col I compared with WT cells, suggesting that TG osteoblasts either made more Col I or were defective in its secretion ($P < 0.0014$, t test). These results indicate that TG osteoblasts have changes in morphology and matrix distribution, which suggests that osteoblast function is altered as a result of transgene expression.

Osteocytes are terminally differentiated cells within the bone matrix that also express osteocalcin and potentially contribute to the changes in bone mass and organization observed in TG animals. Each osteocyte cell body resides in a lacuna within the bone matrix. Multiple filopodia extend from each cell body into canilicular protrusions from the lacuna. The ECM of WT osteocyte lacunae and the associated caniliculi stained positively for Col I (Fig. 8E, arrowheads). This matrix protein was also localized to lacunae surrounding TG osteocytes (Fig. 8F, arrowheads). However, there was no Col I staining of the caniliculi of TG animals, suggesting that these structures were abnormal. A similar difference between TG and WT was seen in staining for LN (data not shown), suggesting that the defects in bone matrix observed in the TG animals were not specific for Col I. To try to determine whether normal filopodia were formed by TG osteocytes, we stained for the gap junction protein connexin 43, a known marker of osteocyte filopodia. Although cell bodies of both normal and TG osteocytes stained positively for connexin 43 (data not shown), the immunofluorescence staining was not sensitive enough to visualize filopodia in either WT or TG mice, and thus we were unable to determine whether the filopodial structures associated with caniliculi were normal in TG osteocytes.

Ninety-day TG Females Have Increased Osteoclast Staining on the Endocranial Surface of Calvaria

Between 35 and 90 days, the murine skeleton grows at a much slower rate. Most bone formation during this period contributes not to net skeletal growth, but to skeletal turnover and remodeling. To determine whether the phenotype observed at 35 days persists during this later phase, we analyzed the parietal bones of WT and TG animals at 90 days. The width of parietal bones of TG males at 90 days was 84% that of WT males, compared with 45% that of WT males at 35 days (Fig. 6B), suggesting that due to continued slow growth and/or remodeling male animals were able to repair or compensate for the original defect in matrix deposition. In contrast, parietal bone width of 90-day TG females was only 53% the value for WT females at 90 days. Thus, the reduced bone width of transgenic females observed at 35 days (Fig. 6B) persisted at 90 days.

During normal remodeling, bone formation by osteoblasts is tightly coupled to its resorption by osteoclasts, a cell of hematopoietic origin that does not express osteocalcin. To determine whether transgene expression was indirectly affecting bone resorption at this later time, calvaria from 90-day TG animals were analyzed by whole-mount staining for tartrate-resistant alkaline phosphatase activity, which specifically stains osteoclasts in bone. In WT animals of both sexes and in TG males there was little staining for TRAP at 90 days (Fig. 9). In contrast, TG females had high levels of TRAP staining on the endocranial surface of the parietal bone (Fig. 9), suggesting that there was an increase in osteoclast number and/or activity on this surface. At 35 days there had been very little staining for osteoclasts on parietal bones of either sex in both WT and TG animals (data not shown), and thus it is unlikely that changes in osteoclast activity contributed to the decreased bone mass observed in TG animals at 35 days. In contrast, the findings at 90 days suggest that there is a second phenotype in TG animals that is sex-specific and characterized by enhanced osteoclast activity on the inner surface of the parietal bone in TG females.

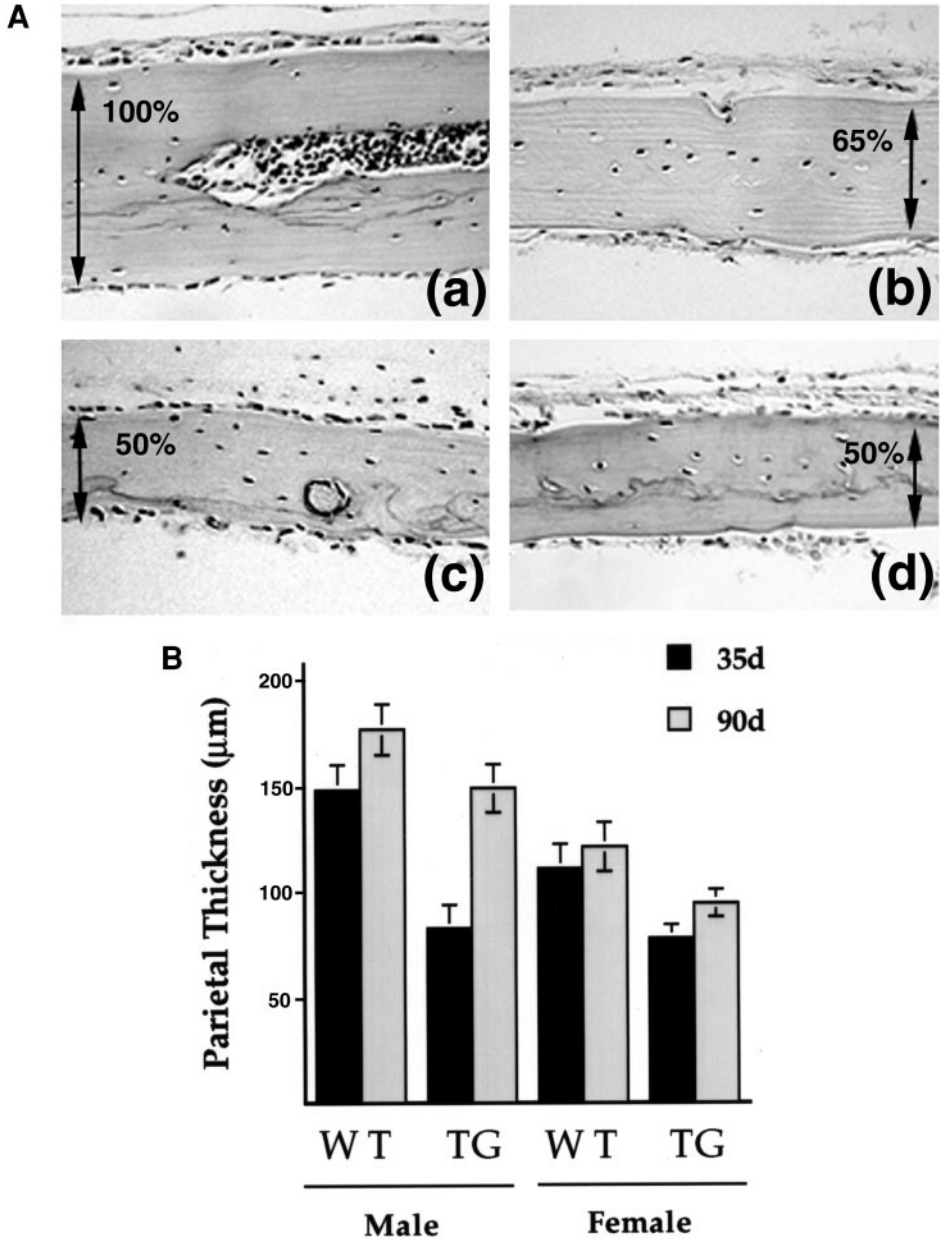

FIG. 6. TG animals have thin parietal bones. (A) Cross sections through the parietal bones of 35-day WT male (a) and female (b) and TG male (c) and female (d) animals. The relative widths of the parietal bones are indicated with arrows; note that WT male (a) is 100%. (B) Quantitative measurements of the thickness of parietal bones from WT and TG animals at 35 and 90 days. The bars denote SEM. The differences between WT and TG males and females are statistically significant (for 35-day data $P < 0.006$; for 90-day data $P < 0.05$, t test). At 35 days, $n = 10$ for TG females, 4 for TG males, 7 for WT females, and 5 for WT males. At 90 days, $n = 5$ for TG females, 4 for WT females, 9 for TG males, and 6 for WT males.

Increased Resorption Results in a Net Loss of Bone

X-ray analysis was used to determine the density of bone over the entire calvarial surface. In both 90-day WT and 90-day TG females, areas of the parietal bone that had high TRAP staining also had decreased radiopacity (compare Figs. 10A and 10C to 10B and 10D). These data indicate that in areas where TRAP staining was high, the resorption of bone by osteoclasts led to a net loss of bone mass. The overall bone mass was much less in TG females compared

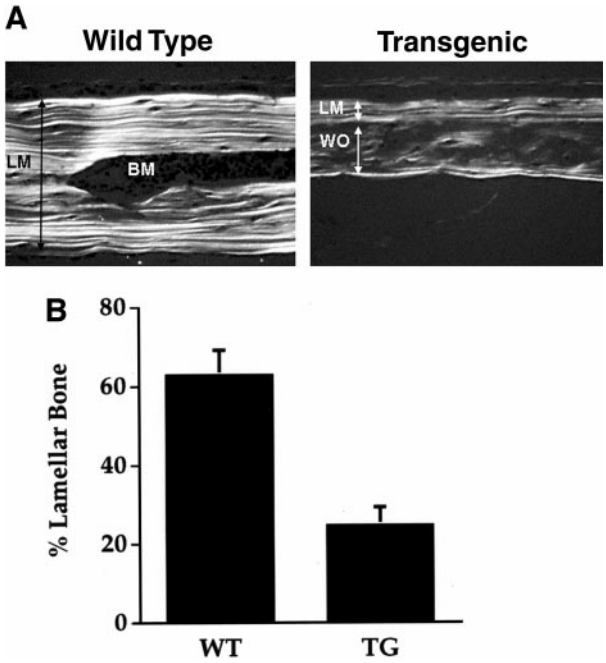

FIG. 7. In TG animals collagen fibrils are disorganized. (A) Cross sections through WT and TG parietal bones viewed through polarized light microscopy. Lamellar (LM) and woven (WO) bone are indicated by arrows. (B) Quantitative measurements of the organization of collagen fibrils in the parietal bone. The bars denote SEM. The differences between WT ($n = 10$) and TG ($n = 11$) are statistically significant ($P < 0.6 \times 10^{-5}$).

with WT females, which correlated with the increased TRAP staining that we observed.

The matrix of bone undergoing resorption is distinct from the matrix of quiescent or forming bone, and this difference can be observed by scanning electron microscopy. Because TRAP staining in TG females was particularly strong above the occipital ridge (Fig. 10C), occipital bones were stripped of cellular and other organic components and the structure of the bone matrix was analyzed by scanning electron microscopy (Fig. 11). In WT females the occipital ridge had a smooth tapering surface (Fig. 11, black arrows, $n = 5$). In contrast, the occipital ridge in TG females was rough and irregular (Fig. 11, white arrows, $n = 5$), presumably due to bone resorption by osteoclasts.

DISCUSSION

The osteoblast is a differentiated cell of mesenchymal origin that produces and organizes most of the proteins present in bone ECM. To better understand the function of integrins in this unique cell type, we targeted expression of a dominant-negative β1 integrin subunit, β1-DN, using the osteocalcin promoter, which is expressed only in mature osteoblasts and osteocytes. β1-DN coded for a chimeric molecule containing the cytoplasmic and transmembrane domains of the β1 integrin subunit, with a truncated portion of the extracellular domain that terminates with an 8-amino-acid epitope tag from HA. Based on our results, we propose that β1-DN expression affects bone metabolism by at least two mechanisms, the first of which occurs when the skeleton is rapidly growing. During this phase of bone development, interfering with integrin function leads to a defect in the formation and organization of bone matrix. Both sexes are affected by the defect initially. However, mature male animals are eventually able to make a near normal amount of bone mass, once the period of rapid growth is completed, while mature females cannot. Increased resorption by osteoclasts in TG female mice, the second aspect of this phenotype involving altered integrin function, appears to render them incapable of fully compensating for the original defect in bone formation.

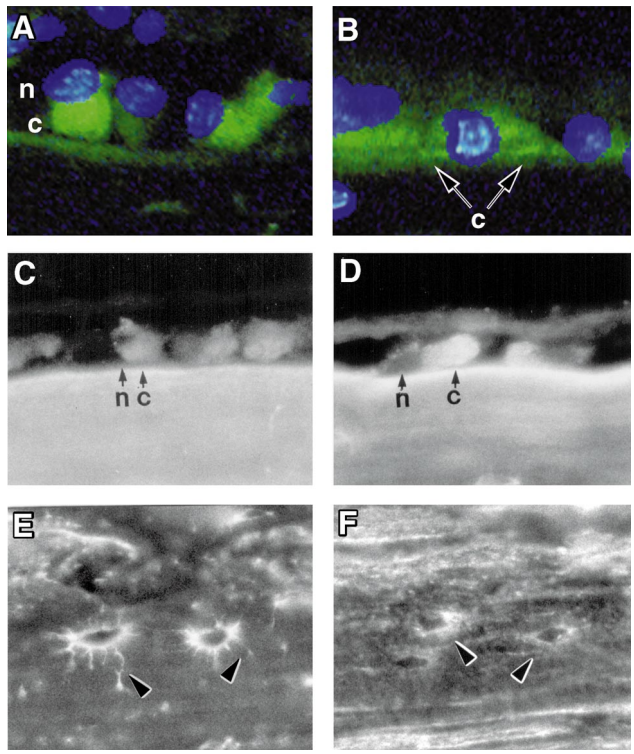

FIG. 8. Osteoblast polarity and secretion of collagen I matrix are altered in TG animals. Cross sections through the parietal bone of 35-day WT (A, C, and E) and TG (B, D, and F) animals stained by IF with antibodies against Col I. (A and B) Most WT osteoblasts are polarized, with the nucleus at the end away from the bone surface (n, nucleus; c, cytoplasm; nuclei are stained by Hoechst). (C and D) TG osteoblasts stain more strongly for intracellular Col I than WT: note also altered cell polarization in TG. (E and F) The caniliculi of osteocytes stain positively for Col I in WT (E) but not in TG (F) samples (arrowheads). Similar differences were seen in preparations stained for LN, FN, and osteopontin (not shown).

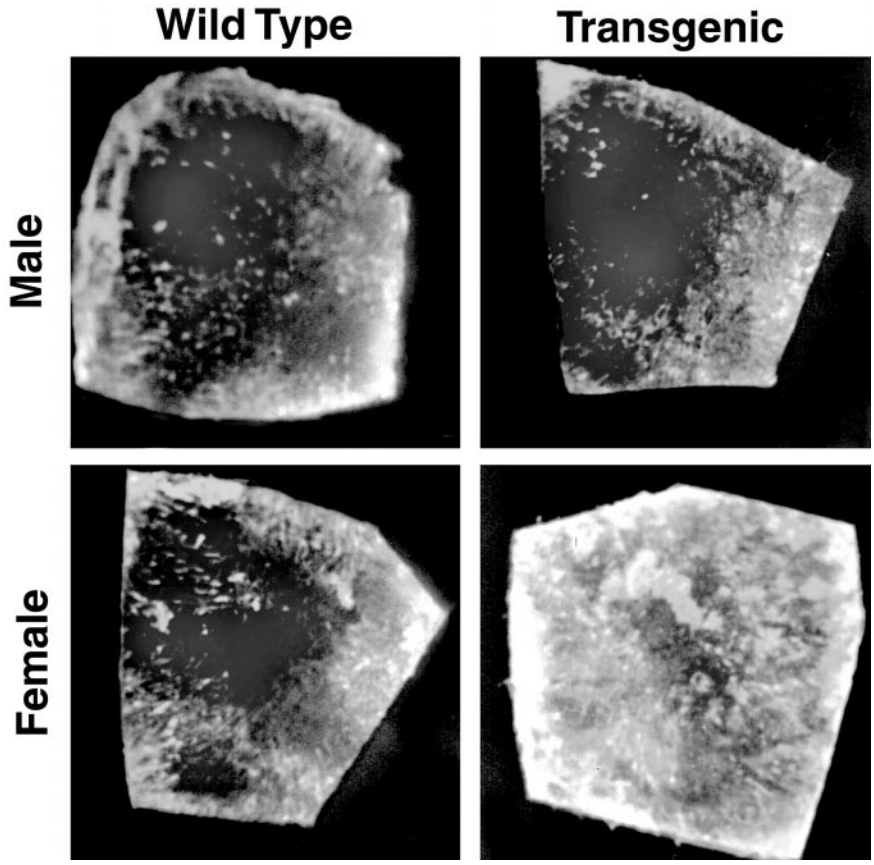

FIG. 9. TG females have increased staining for osteoclasts on the endocranial surface of parietal bones. Parietal bones from 90-day WT and TG animals were fixed and stained for TRAP. The inner, or endocranial, surface of parietal bones from TG females, but not from WT or TG males, stained strongly for TRAP. The color photographs were converted to gray scale and are shown as inverted images so that TRAP-stained bone appears white and unstained bone appears black.

Our results show that growing TG mice of both sexes had decreased bone mass in cortical bone and in the flat bones of the skull. At 35 days, the rate of bone formation in cortical bone was decreased by 60%. Quantitative analysis of the ECM associated with TG osteoblasts showed that active osteoblasts had larger intracellular areas that stained strongly by IF for matrix molecules such as Col I and LN. Although not examined quantitatively, preliminary assessment also suggested that TG osteoblasts were often nonpolar. Taken together, the results shown in Fig. 8 suggest that TG osteoblasts are defective in either the deposition or the organization of matrix and that the defect in osteoblast function may account for the observed decrease in the rate of bone formation.

In TG animals, none of the matrix molecules that we stained for by IF (including Col I, LN, FN, and osteocalcin) stained canilicular structures around TG osteocytes, although staining for these matrix components was very strong in WT caniliculi (shown for Col I, Figs. 8E and 8F). These findings suggest that normal canilicular structures were not formed by osteocytes in TG animals. It is interesting that β1-DN did not affect osteocyte lacunae formation or terminal differentiation. Further functional experiments will be needed to determine what role, if any, integrins have in these processes.

Using primary rat calvarial osteoblast cultures (Moursi *et al.*, 1996, 1997), and the 2T3 mouse osteoblast cell line (Jikko *et al.*, 1999), we showed previously that integrin interactions with FN and Col I are critical for osteoblast differentiation *in vitro*. In those experiments integrin–ECM interactions were perturbed at early stages of osteoblast differentiation, prior to the expression of osteocalcin. Osteocalcin is a tissue-specific marker for fully differentiated osteoblasts and osteocytes (Mikuni-Takagaki *et al.*, 1995), and the promoter is not highly active *in vivo* until 1–2 days before mineralization of the collagen matrix, which is after the initial skeletal template has already been patterned and formed (Ducy *et al.*, 1997). Thus, by driving expression of β1-DN with the osteocalcin promoter, we did not expect to see changes in skeletal patterning or osteoblast differentia-

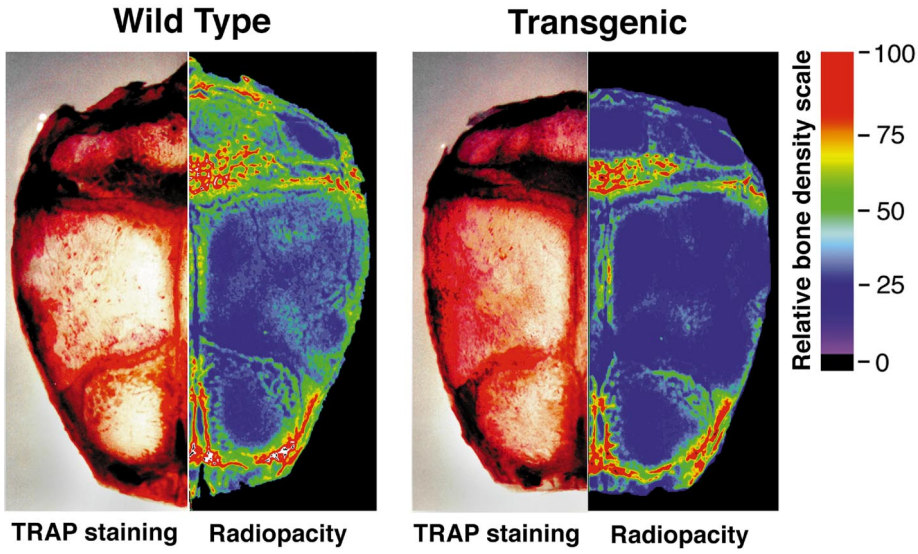

FIG. 10. Areas on calvarial bones with increased TRAP staining have decreased bone mass. Calvarial bones from 90-day female animals were fixed and stained for TRAP (left panels). X-ray analyses of the same bones are shown in mirror image (right panels). Note that areas of high TRAP staining are red and areas of decreased radiopacity are dark blue.

tion because of transgene expression. Osteocalcin is also more highly expressed in cortical bone than in trabecular bone (G. Karsenty, personal communication), and in accordance with this observation, we observed no difference in bone volume or bone formation rate in trabecular bone (data not shown). Future studies using constructs with osteoblast-specific promoters expressed earlier in differentiation will be needed to further elucidate the role of integrins in skeletal patterning and trabecular bone formation.

Constructs similar to β1-DN have been used to perturb integrin function in several different cell types *in vitro* (Chen *et al.*, 1994; Faraldo *et al.*, 1998; LaFlamme *et al.*, 1994; Lukashev *et al.*, 1994; Smilenov *et al.*, 1994). It has thus been shown that β1-DN-type molecules affect different integrin functions depending on the cell type and the levels of expression (Lukashev *et al.*, 1994). At relatively low levels of expression, β1-mediated signaling and matrix organization are affected, whereas high levels of expression of the transgene can cause cells to round up and detach. β1 integrin is highly expressed by osteoblasts (Gohel *et al.*, 1995; Moursi *et al.*, 1996), and high levels of β1-DN expression are required to perturb endogenous β1 integrin function (Lukashev *et al.*, 1994). In TG MSC cultures, expression of β1-DN was high enough to cause cell detachment. However, we did not observe any evidence of cell

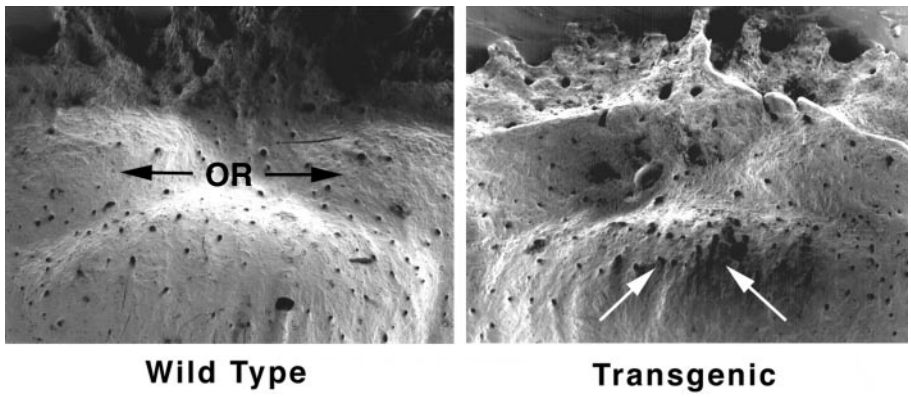

FIG. 11. TG females show deterioration of the occipital ridge (OR). Scanning electron microscopy analysis of 90-day WT and TG occipital bones from females. Images were taken at 30× magnification.

detachment *in vivo*, possibly because of the more complex nature of the ECM or because firmer cell adhesion to this ECM *in vivo* makes cell attachment more difficult to perturb.

Our results are consistent with findings from other systems about the *in vivo* roles of integrins in tissue formation and organization. β1 deficiency in F9 teratocarcinomas interferes with cell polarity and basement membrane assembly and results in changes in the composition of the basement membrane (Sasaki *et al.*, 1998; Stephens *et al.*, 1993). Recently, a chimeric molecule similar to β1-DN, containing the cytoplasmic and transmembrane domains of the β1-integrin chain and the extracellular domain of the T-cell differentiation antigen CD4, was targeted to the mouse mammary gland by the mouse mammary tumor virus MMTV promoter (Faraldo *et al.*, 1998). Targeting of this dominant-negative integrin chain interfered with normal mammary gland development and function. In these TG mice, there were changes in the distribution of LN and β4 integrin from strictly basal to basal and lateral. Here we have shown, using a similar approach, that integrins are directly involved in multiple osteoblast functions, including maintaining cell polarity and regulating the deposition of bone matrix and the formation and/or composition of osteocyte caniliculi (Fig. 12).

In mature animals normal bone mass is maintained by the remodeling of existing bone structures and involves a delicate balance between the activities of bone-forming osteoblasts and matrix-degrading osteoclasts. Increased bone remodeling caused by physiological or environmental factors often results in a net loss of bone (Teitelbaum *et al.*, 1997). In mature (90-day) TG females, we observed an increase in osteoclast staining on the endocranial surface of the parietal bone. The areas of the parietal and occipital bones that showed increased staining also corresponded to areas of decreased bone mass. These findings suggest that the decrease in bone mass observed in 90-day TG females was due to increased resorptive activity by osteoclasts, rather than to a continuation of the original defect in matrix deposition.

TG males did not have increased staining for osteoclasts at 90 days, and the width of the parietal bones was similar to that of WT males. This result suggests that in males, the primary defect in matrix deposition observed at the conclusion of the rapid growth phase (35 days) was compensated for over the period of slow growth and onset of remodeling. At both 35 and 90 days there is no sex difference in expression from the osteocalcin promoter *in vivo* (Frenkel *et al.*, 1997). Thus, the sex differences we observe in the later phenotype (90 days) are most likely due to direct effects of β1-DN expression. As a result, mature females have a decrease in bone mass that results, at least in part, from increased bone resorption by osteoclasts. This finding suggests that integrins might be involved in osteocyte signaling and raises the question of whether integrin signaling through osteoblasts or osteocytes is involved in hormonal regulation of bone remodeling.

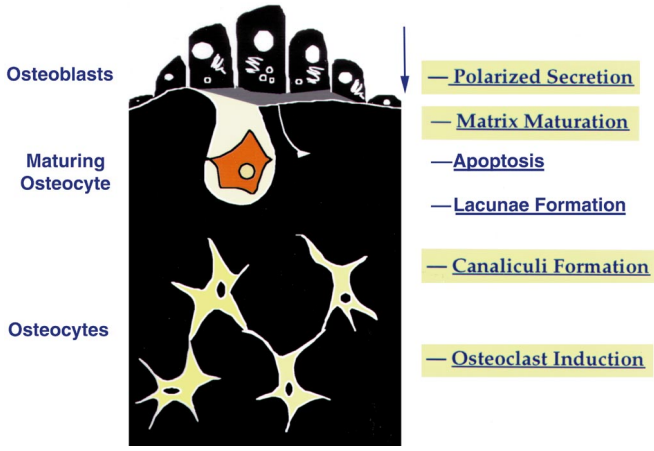

FIG. 12. Model of integrin involvement in osteoblast function. Active osteoblasts are polarized secretory cells localized on forming bone surfaces. Most osteoblasts eventually undergo apoptosis, but a subset of cells terminally differentiates into osteocytes, which are isolated cells that reside within the bone matrix. Each osteocyte occupies a lacuna and extends filopodial processes through caniliculi in the matrix. During terminal differentiation, osteoblast interactions with the ECM change dramatically. To form an osteocyte lacunae, an osteoblast changes from a polarized to a nonpolarized secretory cell. The environment changes from a cell-rich environment to a matrix-rich environment, and cell contact with ECM increases while cell–cell contact decreases. At each stage of osteoblast function there are thus multiple cell–cell and cell–ECM interactions in which integrins may have a role. The functions that are affected by β1-DN expression are highlighted in yellow.

Osteoblasts have multiple complex interactions with ECM that change as an osteoblast differentiates into an osteocyte (Fig. 12). Here we have shown that integrins are required at multiple stages of osteoblast function and that altering integrin function in mature osteoblasts *in vivo* causes changes in osteoblast function that lead to decreased bone mass by at least two different mechanisms.

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