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# Transgenic Mice Overexpressing Insulin-Like Growth Factor Binding Protein-5 Display Transiently Decreased Osteoblastic Function and Osteopenia

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**Skeletal cells synthesize IGFs and their six IGF binding proteins (IGFBP). IGFBP-5 was reported to stimulate bone cell growth *in vitro* and selected parameters of osteoblastic function *in vivo*, but its actual effects on bone formation are not established. We investigated the direct effects of IGFBP-5 on bone remodeling in two lines of transgenic mice overexpressing IGFBP-5 under the control of the osteocalcin promoter. Static and dynamic histomorphometry revealed that IGFBP-5 transgenic mice had a transient decrease in trabecular bone volume secondary to reduced trabecular number and thickness and a transient decrease in bone mineral apposition rate. Osteoblast number was normal, indicating impaired osteoblastic function. Osteoclast number and bone resorption were normal. Total, vertebral, and femoral bone mineral densities were reduced in IGFBP-5 transgenics by 14–27% at 4 wk of age, but not in older animals. Stromal cells expressing the IGFBP-5 transgene displayed decreased expression of alkaline phosphatase, osteocalcin, core binding factor 1, and type I collagen transcripts when compared with cells from wild-type animals. In conclusion, transgenic mice overexpressing IGFBP-5 in the bone microenvironment have a transient decrease in trabecular bone volume, impaired osteoblastic function, and osteopenia. (*Endocrinology* 143: 3955–3962, 2002)**

BONE REMODELING IS a process regulated by hormones and growth factors (1). Skeletal cells synthesize a number of growth factors and their binding proteins. IGF-I and -II are among the most prevalent growth factors secreted by skeletal cells and have important effects on bone formation. IGF-I and -II have modest mitogenic activity for cells of the osteoblastic lineage but are unique among growth factors, because they enhance the differentiated function of the osteoblast (2, 3). In skeletal cells, IGFs can be regulated by changes in their synthesis, receptor binding, and binding proteins. The levels and activity of IGFs are regulated by six IGF binding proteins (IGFBPs), all of which are expressed by the osteoblast (4–7). The precise function of all IGFBPs in skeletal cells is not known. Some, such as IGFBP-1, are important in glucose homeostasis; others, such as IGFBP-2 and -3, are important in the storage and transport of IGFs (6, 7). IGFBP-4 and IGFBP-5 are intriguing because IGFBP-4 often inhibits and IGFBP-5 can stimulate bone cell growth and enhance the effects of IGF-I (8, 9).

In view of its postulated stimulatory effects on bone cell growth, IGFBP-5 has been studied extensively. IGFBP-5 expression is coordinated with that of IGF-I, suggesting important interactions among the two peptides. Agents that stimulate IGF-I mRNA expression, such as IL-6, also stimulate IGFBP-5 transcription (10, 11). IGFBP-5 is secreted intact, and then can be fragmented by the action of matrix metalloproteinases and serine proteinases, and its fragments could have distinct biological activities (10, 12, 13). IGFBP-5 has been reported to stimulate cell proliferation and selected markers of osteoblastic function *in vitro* and *in vivo* (9, 14). It is not certain whether the postulated anabolic effects are due to interactions between IGF-I and IGFBP-5, although direct anabolic effects of IGFBP-5 are possible because they are also observed in cells from IGF-I null mice (14). In addition, interactions of IGFBP-5 with other extracellular matrix proteins, such as vitronectin, modulate the responsiveness to IGF-I and the anabolic effect of IGFBP-5 (15).

Although IGFBP-5 stimulates cell growth and selected parameters of osteoblastic function, such as alkaline phosphatase activity and osteocalcin, its actual effects on bone formation have not been established (14). Knowledge of these effects is necessary since isolated changes in osteocalcin and alkaline phosphatase activity do not necessarily represent changes in bone formation (16). The intent of this study was to investigate the direct effects of IGFBP-5 on bone remodeling. For this purpose, we created transgenic mice overexpressing IGFBP-5 under the control of the osteoblast-specific osteocalcin promoter, and determined their skeletal phenotype by histomorphometric analysis.

## Materials and Methods

### *Osteocalcin/IGFBP-5 construct*

IGFBP-5 was expressed under the control of the osteocalcin promoter so that transcription would occur specifically in cells of the osteoblastic lineage (17). For this purpose, a 1.7-kb fragment of the rat osteocalcin promoter was provided by Dr. R. Derynck (Departments of Growth and Development, University of California at San Francisco, CA) (18). A construct coding for rat IGFBP-5, spanning bp +748 to +1504 of the IGFBP-5 gene, in relationship to the start site of transcription (bp +1), was created (19). The second intron of the rabbit $\beta$-globin gene (0.6 kb) was included between the osteocalcin promoter and IGFBP-5 coding

Abbreviations: BMD, Bone mineral density; BS, bone surface; Cbfa-1, core binding factor 1; d, deoxy; GFP, green fluorescent protein; IGFBP, IGF binding protein.

sequences, which were followed by a 0.2-kb fragment containing polyadenylation coding sequences from the bovine GH gene. The *in vitro* activity of the osteocalcin promoter construct was demonstrated by subcloning the green fluorescent protein (GFP) gene, in place of IGFBP-5, and detection of GFP expression in transiently transfected ROS17/2.8 osteosarcoma cells and MC3T3 osteoblastic cells by fluorescence microscopy (not shown) (20). The ability of the construct to drive IGFBP-5 *in vitro* was confirmed by transiently transfecting the construct into ROS17/2.8 cells and identification of IGFBP-5 transgene mRNA by Northern blot analysis, and protein by Western blot analysis (Fig. 1).

### *Generation of transgenic mice and determination of transgene expression*

Microinjection of linearized DNA into pronuclei of fertilized oocytes from CD-1 outbred albino mice (Charles River Laboratories, Inc., Cambridge, MA), and transfer of microinjected embryos into pseudopregnant mice were carried out at the transgenic facility of The University of Connecticut Health Center (Farmington, CT). Positive founders for osteocalcin driven IGFBP-5 transgene were identified by Southern blot analysis of tail DNA (21). Founder mice were bred to wild-type CD-1 outbred albino mice. Heterozygous and nontransgenic littermates from the F1 and subsequent generations were selected by Southern blotting of genomic DNA. Heterozygous mice were intercrossed to generate a homozygous offspring, which was identified by the intensity of the transgenic band in Southern blots. The results described were obtained from the analysis of two transgenic lines, derived from independent founders. All animal experiments were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

### *Bone histomorphometric analysis*

Static and dynamic histomorphometry was carried out in mice following calcein, 20 mg/kg, injections 7 d and 2 d before the mice were killed by $CO_2$ asphyxiation. Femurs were dissected and fixed in 70% ethanol. After dehydration, bone samples were embedded in methyl methacrylate and 5-$\mu$m sections were cut (Microm, Richard-Allan Scientific, Kalamazoo, MI) and stained with toluidene blue. Static and dynamic parameters of bone structure, bone formation and resorption were measured at a standardized site 195 $\mu$m below the growth plate using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA). Trabecular bone volume/density (BV/TV, %) and relative osteoid volume and surface (%) were calculated, osteoid width ($\mu$m), trabecular thickness (Tb.Th, $\mu$m), spacing ($\mu$m) and number (Tb.N, per mm) were measured. Osteoblast number/trabecular area/mm$^2$, osteoclast surface/bone surface (Oc.S/BS, %) and osteoclast number (Oc.N/BS, mm$^2$) were determined. For dynamic histomorphometry, mineral apposition rate ($\mu$m/d) was measured on unstained sections under UV light, mineralizing surface was expressed as a percentage of total BS (MS/BS %), and bone formation rate ($\mu$m$^3$/$\mu$m$^2$/d) was calculated. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (22).

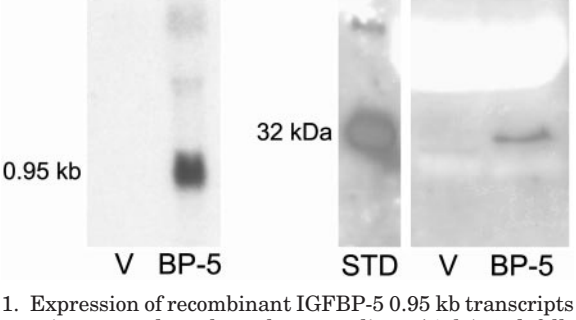


FIG. 1. Expression of recombinant IGFBP-5 0.95 kb transcripts (*left*) and protein secreted to the culture medium (*right*) 6 d following transient transfection of ROS17/2.8 cells with osteocalcin-IGFBP-5 (BP-5) or osteocalcin GFP (control vector, V) constructs.

### *X-ray analysis and bone mineral density*

Radiography was performed on mice anesthetized with tribromoethanol (Sigma, St. Louis, MO) on a Faxitron x-ray system (model MX 20, Faxitron X-Ray Corp., Wheeling, IL). Total, femoral, and vertebral bone mineral density (BMD) was measured on anesthetized mice using the PIXImus small animal dual energy x-ray absorptiometry system (GE Medical Systems/Lunar Corp., Madison, WI) (23). Calibrations were performed with a phantom of a defined value, and quality assurance measurements were performed daily. The coefficient of variation for total BMD is less than 1% (n = 9).

### *Bone marrow stromal cell cultures*

Femurs were aseptically removed from 7-wk-old mice, following $CO_2$ asphyxiation, and dissected free of adhering tissues. The ends were removed and cells were recovered by centrifugation (24). Cells were plated at a density of $10^6$ cells/cm$^2$ and cultured in $\alpha$-MEM (Invitrogen, Rockville, MD) containing 15% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) at 37 C in a humidified 5% $CO_2$ incubator. Half the volume of the culture medium was replaced, after 4 d of culture, with fresh medium. At confluence (7–9 d of culture), the medium was changed to $\alpha$-MEM supplemented with 15% fetal bovine serum, 50 $\mu$g/ml ascorbic acid and 8 mM $\beta$-glycerophosphate (mineralizing medium) (Sigma). The medium was replaced twice a week for the duration of the culture, and cells were harvested 3 d after the last change of medium. For cytochemical analysis, cells were fixed with 4.5 mM citric acid, 2.25 mM sodium citrate, 65% acetone, and 8% formalin and stained for the presence of mineralized nodules using 2% alizarin red (Sigma).

### *Northern blot analysis*

Total femoral and calvarial bone RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction method (25) followed by purification with an RNeasy column (QIAGEN, Valencia, CA). Total cellular RNA was isolated using an RNeasy kit per manufacturer's instructions (QIAGEN). RNA was quantitated by spectrometry, and equal amounts of RNA were loaded on a formaldehyde-agarose gel following denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA, and to confirm equal RNA loading of the samples. The RNA was blotted onto GeneScreen Plus charged nylon (Perkin-Elmer, Norwalk, CT), and uniformity of transfer was confirmed by revisualization of ethidium bromide-stained ribosomal RNA. A 2.5-kb rat alkaline phosphatase cDNA (Merck and Co., West Point, PA), a 2.5-kb core binding factor 1 (Cbfa-1) murine cDNA (Ito, Y., Kyoto, Japan), a 1.6-kb rat type I collagen cDNA (Kream, B., Farmington, CT), a 0.5-kb osteocalcin genomic rat DNA containing no sequence homology with the osteocalcin promoter driving IGFBP-5 (Lian, J., Worcester, MA), a 330-bp rat IGFBP-5 cDNA (Shimasaki, S., La Jolla, CA), and a 0.75-kb 18S cDNA (American Type Culture Collection, Manassas, VA) were purified by agarose gel electrophoresis (19, 26–28). DNAs were labeled with deoxy (d) CTP and [$\alpha$-$^{32}$P]-dATP (50 $\mu$Ci each at a specific activity of 3000 Ci/mmol; Perkin-Elmer) using the random hexanucleotide-primed second-strand synthesis method (29) or labeled with Ready-To-Go DNA labeling beads (dCTP) kit (Amersham Pharmacia Biotech, Piscataway, NJ), in accordance with manufacturer's instructions. Hybridizations were carried out at 42 C for 16–72 h, followed by two posthybridization washes at room temperature for 15 min in 1× saline sodium citrate (SSC), and a wash at 65 C for 20–30 min in 0.5× or 1× SSC. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak Co., Rochester, NY), employing Cronex Lighting Plus (Perkin-Elmer) or Biomax MS (Eastman Kodak Co., Rochester, NY) intensifying screens. Relative hybridization levels were determined by densitometry. Northern analyses shown are representative of two to three samples.

### *RT-PCR*

One microgram of deoxyribonuclease I-treated total RNA from stromal cell and bone extracts was reverse-transcribed with murine Moloney leukemia virus reverse transcriptase (Invitrogen) in the presence of 20 $\mu$M reverse primer corresponding to an 18-bp fragment of bovine GH

polyadenylation sequence present in the IGFBP-5 vector. One-microliter of reverse transcription reaction was amplified by 30 cycles of PCR at 64 C annealing temperature in the presence of 20 $\mu$M 5′ to 3′ primer spanning bp +459 to +479 of IGFBP-5 coding sequence, [$\alpha$-$^{32}$P]-dATP and 2.5 U of *Taq* polymerase to yield a product of a predicted size of 416 bp. The products of the PCR were resolved by electrophoresis on a 6% polyacrylamide gel and exposed to Kodak X-AR5 film overnight and developed.

### *Western immunoblot analysis*

Culture medium from transfected ROS 17/2.8 cells, concentrated under vacuum by centrifugation, protein extracts from homogenized femurs or serum aliquots from control and transgenic mice were fractionated by polyacrylamide gel electrophoresis (30). Proteins were transferred to Immobilon P membranes (Millipore Corp., Bedford, MA), blocked with 2% BSA and exposed to rabbit antiserum against human IGFBP-5 (Upstate Biotechnology, Inc., Lake Placid, NY) overnight. Blots were exposed to goat antirabbit IgG antiserum conjugated to horseradish peroxidase, and developed with a chemiluminescence detection reagent (DuPont, Wilmington, DE) (31). IGFBP-5 was identified by comigration with recombinant purified human IGFBP-5 (Upstate Biotechnology, Inc.).

### *Serum IGF-I, osteocalcin, and alkaline phosphatase activity*

Serum immunoreactive IGF-I was measured, following removal of IGFBPs by acidification and silica chromatography, using a commercial RIA kit for human IGF-I in accordance with manufacturer's instructions (DiaSorin, Inc., Stillwater, MN). Values are expressed in ng/ml of human IGF-I equivalents. Serum immunoreactive osteocalcin was measured using a commercial RIA kit in accordance with manufacturer's instructions (Biomedical Technologies, Inc., Stoughton, MA). Values are expressed in ng/ml. Serum alkaline phosphatase activity was measured using an Ortho Clinical Diagnostic (Rochester, NY) enzymatic assay that determines the conversion of p-nitrophenyl phosphate to p-nitrophenol. Values are expressed in international units per liter.

### *Statistical analysis*

Data are expressed as means ± SEM. Statistical significance for BMD and histomorphometric and serum-levels data were determined by Student's *t* test.

## Results

### *Generation of transgenic mice and examination of transgene expression*

Two founders expressing the IGFBP-5 transgene were generated. A male (line 1) expressed 4 to 6 copies of the transgene, as estimated by Southern blot analysis, and was used to generate a homozygous offspring expressing 8–10 copies of the transgene. This line was selected for extensive analysis. A second founder, a female (line 2), expressed 16–18 copies of the transgene, and was used to generate heterozygous mice, but attempts to generate a homozygous offspring were unsuccessful. This could suggest that high levels of IGFBP-5 expression may compromise viability. Mice of line 1 were of normal weight and the length of their femurs was not different from wild-type controls. However, heterozygous mice of line 2 at 4 wk of age were 70% the weight of their wild-type littermates and their femurs were (mean ± SEM; n = 13) 11.3 ± 0.3 mm and were shorter than wild-type controls, which were 13.1 ± 0.2 mm (n = 25), $P < 0.05$. Northern blot analysis was used to determine IGFBP-5 overexpression in bones from transgenic and wild-type control mice. IGFBP-5 transcripts of 0.95 kb, the predicted size of the IGFBP-5 transgene mRNA, were detected only in RNA extracted from bones of transgenic mice (Fig. 2, *upper panel*). High levels of transgene mRNA were detected in 4-wk-old transgenic mice, coinciding with the high level of activity of the osteocalcin promoter (17). IGFBP-5 mRNA expression in bone extracts declined with age, in accordance with a decline in the reported activity of the osteocalcin promoter. To confirm that the overexpression of IGFBP-5 was due to expression of the transgene in bone, RT-PCR was carried out in RNA extracted from bones of IGFBP-5 transgenic mice and wild-type controls. RT-PCR demonstrated the presence of IGFBP-5 transgene in bone from both transgenic lines and not in that from wild-type animals (Fig. 2, *middle* and *right lower panels*). To

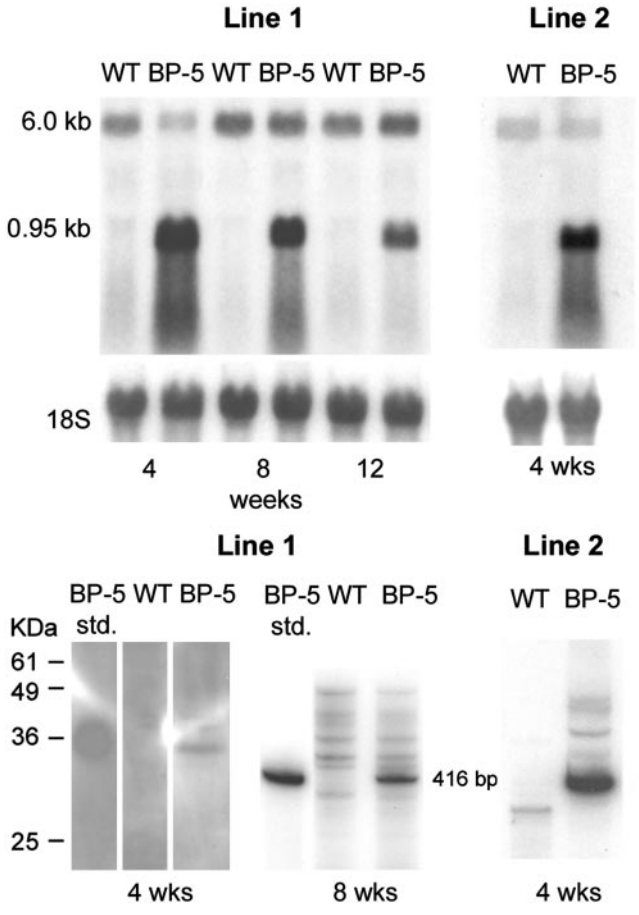


FIG. 2. Expression of IGFBP-5 transgene mRNA by Northern blot analysis (*upper panel*) and by RT PCR (*middle* and *right lower panels*) and expression of IGFBP-5 by Western blot analysis (*left lower panel*) in extracted bone from IGFBP-5 transgenic and wild-type control mice. In the *upper panel*, 10 $\mu$g of total RNA were extracted from bones of homozygous IGFBP-5 transgenic (line 1, *left*) and from age matched wild-type control mice at 4–12 wk of age and from bones of heterozygous IGFBP-5 transgenic mice (line 2, *right*) and wild-type littermates at 4 wk of age. RNA was resolved by gel electrophoresis, transferred to a nylon membrane and hybridized with [$\alpha$-$^{32}$P]-labeled rat IGFBP-5 cDNA. The blot was stripped and rehybridized with 18S cDNA. In the *left lower panel*, 30 $\mu$g of protein were extracted from bones of 4-wk-old homozygous IGFBP-5 transgenic (line 1) and from wild-type control mice, resolved by gel electrophoresis, and incubated with an IGFBP-5 antibody. In the *middle* and *right lower panels*, 1 $\mu$g of RNA from bones of 8-wk-old homozygous (line 1, *left*) and 4-wk-old heterozygous (line 2, *right*) transgenics and wild-type age-matched and littermate controls was reverse transcribed, amplified by PCR, in the presence of [$\alpha$-$^{32}$P]-dATP, resolved by gel electrophoresis, and visualized by autoradiography.

**TABLE 1.** Femoral trabecular bone histomorphometry in 5-wk-old transgenic overexpressing IGFBP-5 and wild-type control mice

| | Line 1 | | Line 2 | |
|---|---|---|---|---|
| | Wild-type | IGFBP-5 | Wild-type | IGFBP-5 |
| Trabecular relative bone volume (%) | 27.0 ± 1.3 | 16.6 ± 1.3[a] | 26.7 ± 3.2 | 15.3 ± 2.2[a] |
| Relative osteoid volume (%) | 0.66 ± 0.12 | 1.05 ± 0.10 | 1.27 ± 0.27 | 1.28 ± 0.24 |
| Osteoid width ($\mu$m) | 0.76 ± 0.03 | 0.95 ± 0.12 | 0.78 ± 0.09 | 0.72 ± 0.09 |
| Relative osteoid surface (%) | 14.3 ± 2.0 | 23.4 ± 5.0 | 21.2 ± 2.6 | 18.3 ± 2.2 |
| Number of osteoblasts/trabecular area (per mm$^2$) | 145 ± 16 | 150 ± 25 | 216 ± 20 | 149 ± 30 |
| Eroded surface/trabecular bone surface (%) | 6.68 ± 1.8 | 6.42 ± 1.4 | 11.2 ± 2.2 | 9.2 ± 1.7 |
| Number of osteoclasts/trabecular bone area (per mm$^2$) | 24 ± 5.3 | 17 ± 3.6 | 45 ± 7.7 | 29 ± 6.0 |
| Trabecular thickness ($\mu$m) | 32.1 ± 1.4 | 26.7 ± 1.0[a] | 26.4 ± 2.1 | 21.6 ± 1.8 |
| Trabecular spacing ($\mu$m) | 87.0 ± 3.9 | 137.5 ± 8.2[a] | 77.7 ± 9.5 | 128.8 ± 17.3[a] |
| Trabecular number (per mm) | 8.5 ± 0.3 | 6.1 ± 0.3[a] | 9.9 ± 0.7 | 6.9 ± 0.6[a] |
| Mineral apposition rate ($\mu$m/d) | 1.13 ± 0.07 | 0.81 ± 0.11[a] | 0.90 ± 0.02 | 0.39 ± 0.016[a] |
| Mineralizing surface (%) | 11.6 ± 1.1 | 14.0 ± 2.0 | 11.4 ± 0.7 | 9.1 ± 3.8 |
| Bone formation rate ($\mu$m$^3$/$\mu$m$^2$/d) | 0.12 ± 0.001 | 0.11 ± 0.02 | 0.10 ± 0.004 | 0.06 ± 0.03[a] |

Bone histomorphometry was carried out on femurs from 5-wk-old IGFBP-5 homozygous (line 1) and heterozygous (line 2) transgenics and wild-type age-matched and littermate controls, respectively. Values are means ± SEM (n = 5–8).

[a] Significantly different from control, $P < 0.05$.

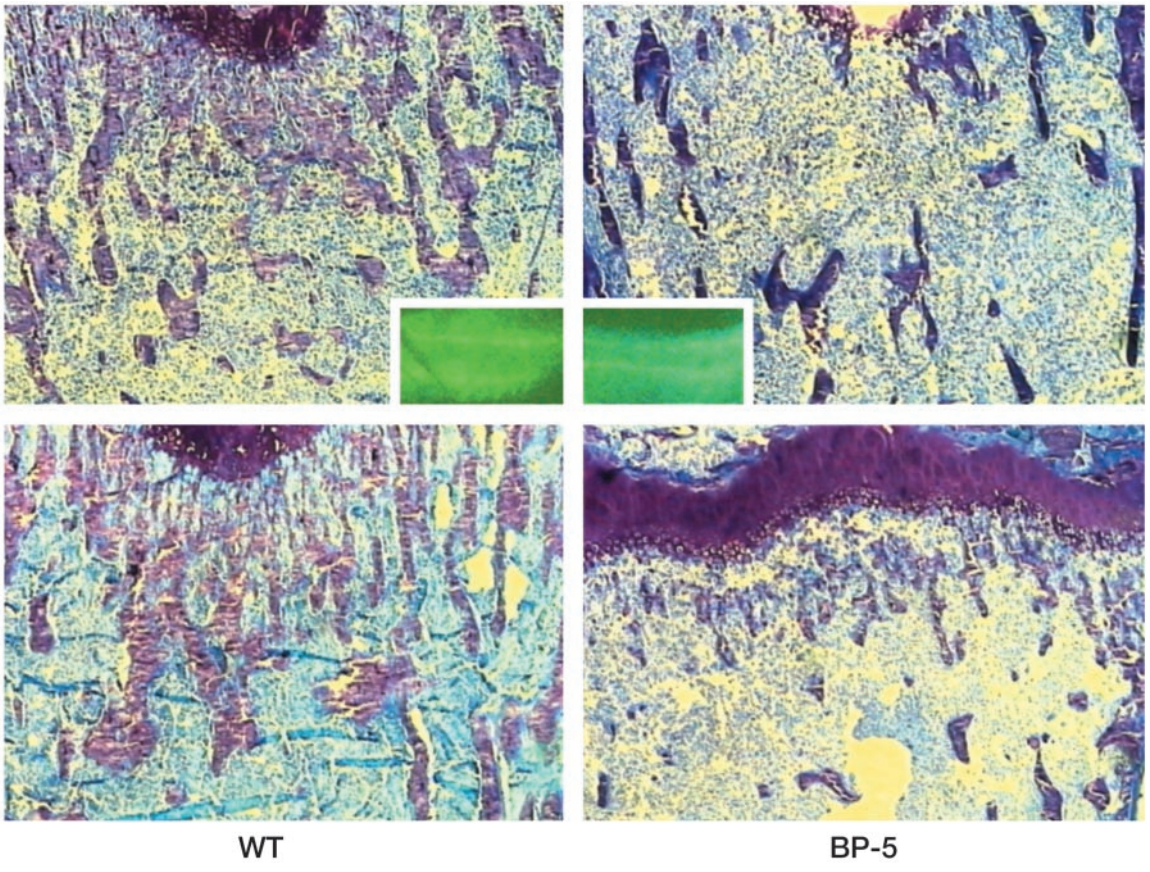


FIG. 3. Representative undecalcified sections of distal femurs stained with toluidene blue from a 5-wk-old homozygous transgenic overexpressing IGFBP-5 (line 1) and age matched wild-type (WT) control (*upper panels*), and a 5-wk-old heterozygous transgenic overexpressing IGFBP-5 (line 2) and WT littermate control (*lower panels*) (×25 magnification). *Insets in upper panels* demonstrate mineralization fronts following dual calcein labeling.

confirm that transgenic mice overexpressed IGFBP-5 in bone, Western immunoblot analysis of extracted femurs was performed and demonstrated detectable levels of IGFBP-5 in 4-wk-old transgenic (line 1) and not in wild-type controls (n = 3) (Fig. 2, *left lower panel*).

*Static and dynamic histomorphometry*

Static and dynamic histomorphometric measurements were made at the distal femur from IGFBP-5 homozygous mice (line 1) and heterozygous mice (line 2) and compared with age-matched and littermate wild-type controls (Table 1,

**TABLE 2.** Selected histomorphometric parameters in 5- to 24-wk-old transgenic overexpressing IGFBP-5 and wild-type control mice

| Weeks | Trabecular bone volume (%) | | Trabecular thickness ($\mu$m) | | Trabecular number (per mm) | | Mineral apposition rate ($\mu$m/d) | | Mineralizing surface/ bone surface % | | Bone formation rate/$\mu m^3/\mu m^2/d$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WT | BP-5 | WT | BP-5 | WT | BP-5 | WT | BP-5 | WT | BP-5 | WT | BP-5 |
| 5 | 27.1 ± 1.3 | 16.6 ± 1.3[a] | 32.1 ± 1.4 | 26.7 ± 1.0[a] | 8.5 ± 0.3 | 6.1 ± 0.3[a] | 1.13 ± 0.07 | 0.81 ± 0.11[a] | 11.6 ± 1.1 | 14.0 ± 2.0 | 0.12 ± 0.01 | 0.11 ± 0.02 |
| 9 | 20.2 ± 2.0 | 17.0 ± 1.6 | 34.3 ± 2.2 | 40.4 ± 2.1[a] | 5.8 ± 0.4 | 4.1 ± 0.3[a] | 0.81 ± 0.03 | 1.01 ± 0.05[a] | 9.3 ± 0.9 | 11.5 ± 0.5 | 0.08 ± 0.01 | 0.11 ± 0.04[a] |
| 24 | 16.8 ± 2.3 | 14.7 ± 1.5 | 30.3 ± 1.7 | 34.8 ± 2.3 | 5.4 ± 0.4 | 4.1 ± 0.2[a] | 0.59 ± 0.09 | 0.50 ± 0.08 | 3.5 ± 0.8 | 4.0 ± 0.7 | 0.02 ± 0.01 | 0.02 ± 0.01 |

Bone histomorphometry was carried out on femurs from 5- to 24-wk-old IGFBP-5 homozygous transgenics (BP-5, line 1) and wild-type (WT) age-matched controls. Values are means ± SEM (n = 6–8).

[a] Significantly different from control, $P < 0.05$.

Fig. 3). At 5 wk of age, transgenic mice had about 40% less trabecular bone volume, and this appeared to be secondary to a reduction in trabecular number and thickness, although trabecular thickness was significantly lower in line 1, but not in line 2, $P < 0.056$. The number of osteoblasts was unchanged, but bone mineral apposition rate was 30–50% lower in transgenic than in wild-type mice, as determined by double calcein labeling, indicating impaired osteoblastic function (Table 1, Fig. 3). Bone formation rate was reduced in line 2, but not in line 1. Osteoclast number and bone resorption were not altered in transgenic IGFBP-5 mice. Consistent with the activity of the osteocalcin promoter, most of the phenotypic impact of IGFBP-5 overexpression, as determined by bone histomorphometry, occurred in the first weeks of life (Table 2). At 9 wk of age there was a modest, possibly compensatory, increase in trabecular thickness and mineral apposition rate in IGFBP-5 transgenics. The effect was transient, whereas a decrease in trabecular number was sustained for 9–24 wk (Table 2).

### *X-rays and bone mineral density*

Contact radiography of transgenic mice overexpressing IGFBP-5 from both lines did not reveal the presence of obvious osteopenia or fractures at 4 wk of age (not shown). Transgenic mice overexpressing IGFBP-5 had significant decreases in BMD when compared with wild-type controls (Table 3). At 4 wk of age, the decrease in BMD in transgenic mice was 16% for total BMD, 19–27% for femoral BMD and 14 to 22% for vertebral BMD in both transgenic lines examined. As observed with changes in bone formation, the effect on BMD was transient and not sustained after 8–24 wk (Table 3).

### *Serum IGFBP-5, IGF-I, osteocalcin, and alkaline phosphatase activity*

The overexpression and impact of IGFBP-5 appeared circumscribed to the bone microenvironment of IGFBP-5 transgenic mice. At 4 wk of age, serum levels of IGFBP-5 were virtually undetectable by Western blot analysis in both wild-type and IGFBP-5 transgenics (not shown), and there was no difference in serum IGF-I concentrations. Serum IGF-I levels were (mean ± SEM, n = 7) 263 ± 14 ng/ml (35 ± 2 nM) in wild-type mice, and 257 ± 14 ng/ml (34 ± 2 nM) in IGFBP-5 transgenics (line 1). IGFBP-5 transgenics did not display changes in serum levels of osteocalcin or alkaline phosphatase. At 4 wk of age, the serum levels of osteocalcin were (mean ± SEM, n = 7) 108 ± 6 ng/ml in wild-type and 96 ± 12 ng/ml in IGFBP-5 transgenic (line 1) mice, and the serum alkaline phosphatase activity was 223 ± 8 IU/liter in wild-type and 204 ± 10 IU/liter in IGFBP-5 transgenics.

### *Stromal cell cultures and osteoblastic gene markers*

Northern blot analysis of RNA extracted from mouse femurs and calvariae did not reveal a consistent decrease in the expression of the osteoblastic gene markers alkaline phosphatase, osteocalcin, Cbfa-1, and type I collagen in homozygous transgenic mice (line 1) compared with wild-type control mice (not shown). To investigate further the impact of

**TABLE 3.** Bone mineral density of 4- to 24-wk-old IGFBP-5 transgenic and wild-type mice

| Weeks | Total BMD g/cm$^2$ | | Femoral BMD g/cm$^2$ | | Vertebral BMD g/cm$^2$ | |
|---|---|---|---|---|---|---|
| | WT | BP-5 | WT | BP-5 | WT | BP-5 |
| Line 1 | | | | | | |
| 4 | 0.043 ± 0.001 | 0.036 ± 0.001[a] | 0.059 ± 0.001 | 0.048 ± 0.001[a] | 0.046 ± 0.001 | 0.036 ± 0.001[a] |
| 8 | 0.052 ± 0.001 | 0.052 ± 0.001 | 0.069 ± 0.003 | 0.072 ± 0.002 | 0.059 ± 0.002 | 0.064 ± 0.002 |
| 12 | 0.056 ± 0.001 | 0.056 ± 0.001 | 0.077 ± 0.002 | 0.074 ± 0.001 | 0.062 ± 0.002 | 0.068 ± 0.002 |
| 24 | 0.062 ± 0.004 | 0.058 ± 0.001 | 0.083 ± 0.004 | 0.076 ± 0.001 | 0.072 ± 0.005 | 0.071 ± 0.003 |
| Line 2 | | | | | | |
| 4 | 0.038 ± 0.001 | 0.032 ± 0.001[a] | 0.052 ± 0.001 | 0.038 ± 0.002[a] | 0.037 ± 0.001 | 0.032 ± 0.001[a] |

Total, femoral, and vertebral BMD was obtained from 4- to 24-wk-old IGFBP-5 transgenic (BP-5) homozygous (line 1) and from 4-wk-old heterozygous (line 2) and from wild-type (WT) age-matched and littermate controls. Values are means ± SEM (n = 4–20).

[a] Significantly different from control, $P < 0.05$.

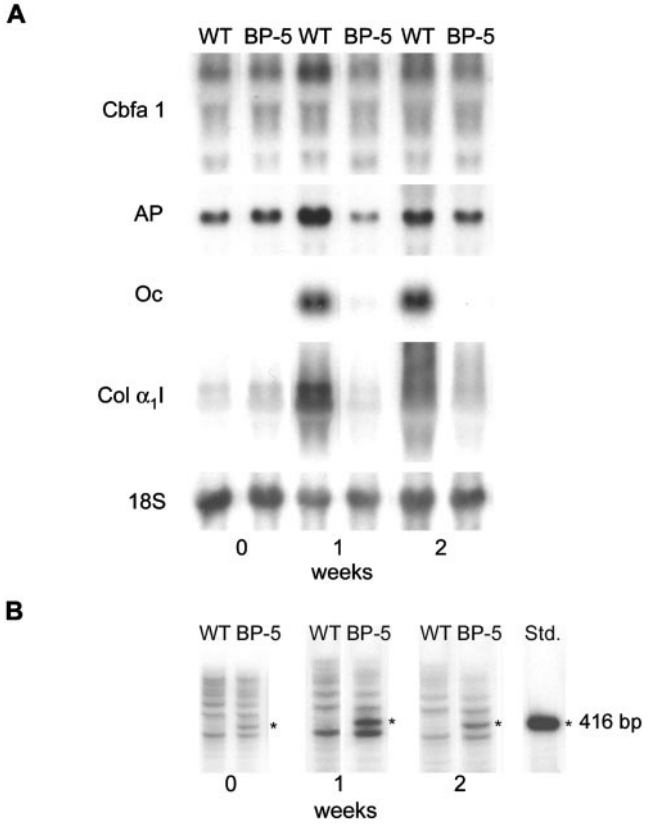


FIG. 4. Northern blot analysis showing the expression of Cbfa-1, alkaline phosphatase (AP), osteocalcin (Oc), and type I collagen (Col $\alpha_1$I) mRNA (*upper panel*) and RT-PCR showing expression of IGFBP-5 transgenic (*lower panel*) in stromal cells from homozygous IGFBP-5 transgenic (line 1) and from age-matched wild-type control mice cultured to confluence (0 wk) and 1 and 2 wk following confluence. On the *upper panel*, 10 $\mu$g of total RNA were resolved by gel electrophoresis, transferred to a nylon membrane, and sequentially hybridized with [$\alpha$-$^{32}$P]-labeled Cbfa-1, alkaline phosphatase, osteocalcin, type I collagen, and 18S DNA. On the *lower panel*, 1 $\mu$g of RNA was reverse transcribed, amplified by PCR in the presence of [$\alpha$-$^{32}$P]-dATP, resolved by gel electrophoresis, and visualized by autoradiography. *, Denotes 416-bp predicted PCR product.

IGFBP-5 on osteoblastic function, stromal cells from 7-wk-old homozygous transgenic mice overexpressing IGFBP-5 (line 1) and from age matched wild-type control mice were cultured for up to 2 wk following confluence. Stromal cells from IGFBP-5 transgenic, but not from wild-type mice, expressed the IGFBP-5 transgene, as determined by RT-PCR, at confluence and 1 and 2 wk after confluence (Fig. 4). Mineralized nodule formation, as determined by alizarin red staining, was reduced in stromal cell cultures from transgenic mice (not shown). Northern blot analysis confirmed impaired osteoblastic maturation or function in stromal cells from IGFBP-5 transgenic mice, and revealed decreased expression of alkaline phosphatase, osteocalcin, Cbfa-1, and type I collagen transcripts, relative to their expression in cultures from wild-type mice (Fig. 4). Except for osteocalcin and type I collagen, the decrease was mostly apparent 1 wk after confluence, when the expression of the IGFBP-5 transgene was more prominent. As the cells from wild-type mice matured and mineralized, there was a decline in Cbfa-1, alkaline phosphatase, and type I collagen transcripts, also contributing to a lesser difference in transcript expression between cells from wild-type and transgenic mice 2 wk after confluence.

## Discussion

Our findings demonstrate that transgenic mice overexpressing IGFBP-5 under the control of the osteocalcin promoter develop decreased trabecular bone volume and osteopenia. The skeletal phenotype, as determined by histomorphometry, is evident at 4–5 wk of age, a time of marked expression of the osteocalcin promoter, but it was not observed in older animals. This is probably because of a decline in the activity of the osteocalcin promoter as animals age, as evidenced by the lesser degree of IGFBP-5 transgene overexpression in older animals (17). The decrease in trabecular bone observed in IGFBP-5 transgenic mice appeared to be secondary to decreased osteoblastic function because osteoblast and osteoclast number were not affected, there was a decrease in mineral apposition rate, and there was no increase in bone resorption. A decrease in selected markers of osteoblastic function was observed in stromal cells from IGFBP-5 transgenics. This effect was mostly observed 1 wk following confluence, when the expression of IGFBP-5 transgene in stromal cells was more prominent, and before the expected decline in osteoblastic gene markers observed with the terminal differentiation and mineralization of wild-type cells.

The mechanism of the impaired osteoblastic function in IGFBP-5 transgenics could involve the binding and sequestering of IGF-I and II by IGFBP-5 in the bone microenvironment. This would be in agreement with the known effects of IGFs on bone formation. IGFs have modest mitogenic activity

in bone and have a more prevalent impact on the differentiated function of the osteoblast as manifested by increased collagen expression *in vitro* and increased mineral apposition rates in transgenic mice overexpression IGF-I (2, 3). Because IGFBP-5 transgenics have decreased mineral apposition rates, the mechanism is likely sequestration of IGF-I. However, IGFBP-5 effects independent of IGFs are possible and were not excluded. These studies also demonstrate that a decrease in trabecular bone volume and osteoblastic function by IGFBP-5 overexpression can translate into significant and generalized osteopenia in the mouse, as determined by BMD.

Previous work has shown that IGFBP-5 stimulates bone cell growth *in vitro* and selected parameters of osteoblastic function *in vivo* (9, 14, 32). Increases in bone formation *in vivo* have been explored and observed in ovariectomized mice but are more evident with the administration of the truncated IGFBP-5 1–169 form than with intact IGFBP-5 (33). The present results could be considered inconsistent with the reported *in vivo* effects following the systemic administration of IGFBP-5 to mice on serum levels of osteocalcin and alkaline phosphatase activity (14). However, by themselves these changes do not necessarily represent changes in bone formation, and they were not confirmed by bone histomorphometric analysis. Furthermore, animals with null mutations for the osteocalcin gene display increased, and not decreased, bone formation confirming that serum markers are not sufficient to establish the presence of an anabolic effect (16). It is possible that the differences in the results observed are related to different strains of mice examined because anabolic effects and changes in bone mass are highly dependent on strain (34). Another possibility is that the inhibitory effects of IGFBP-5 found in transgenic mice are the result of excessive IGFBP-5 in the bone microenvironment, and sequestering of IGFs, and at lower levels IGFBP-5 might be anabolic. Similar discrepancies have been reported for TGF-$\beta$. *In vivo* administration of TGF-$\beta$ can result in increased bone formation, whereas transgenic mice overexpressing TGF-$\beta$ under the control of the osteocalcin promoter develop osteopenia (18, 35). It is, however, important to note that TGF-$\beta$ inhibits the osteoblastic differentiation induced by bone morphogenetic protein-2, indicating direct inhibitory actions of TGF-$\beta$ on cells of the osteoblastic lineage and confirming the results observed in TGF-$\beta$ transgenics (36). The validity of the transgenic model used also can be substantiated by the demonstration of an increase in bone formation by overexpressing IGF-I under the control of the osteocalcin promoter, and confirmation of the known anabolic actions of IGF-I *in vitro* (2, 3).

Confirming the observations *in vivo*, stromal cells from transgenic mice overexpressing IGFBP-5 did not fully differentiate into cells expressing the osteoblastic phenotype. However, it is possible that, due to delayed activation of the osteocalcin promoter, stromal cells underwent partial differentiation and that IGFBP-5, by trapping IGFs, inhibited the differentiated function of partially mature cells. The possibility that IGFBP-5 has no impact on immature cells is in accordance with the known *in vitro* effects of IGF-I on human stromal cells. In this culture system, IGF-I does not alter the differentiation of immature stromal cells into mature cells, but stimulates osteoblast phenotypic markers following cell maturation (37).

The dual inhibitory and stimulatory effects of some IGFBPs are not limited to IGFBP-5. Previous studies have demonstrated that under selected conditions IGFBP-4, which is an inhibitory binding protein, can stimulate parameters of osteoblastic function (8, 38). Consequently, it is possible that both IGFBP-4 and 5 can have stimulatory and inhibitory effects on bone cell function. Recently, the amino terminal fragment of IGFBP-5 1–169 was shown to have anabolic effects in bone *in vivo* (33). This would suggest that IGFBP-5 fragments and not intact IGFBP-5 are anabolic and that under conditions of IGFBP-5 overexpression, necessary and controlled protein fragmentation might not have occurred. Another possibility could be lack of association to critical extracellular matrix proteins, such as vitronectin, under conditions of IGFBP-5 overexpression because these associations enhance the anabolic effect of IGF-I in the presence of IGFBP-5 (15).

In conclusion, our studies demonstrate that IGFBP-5 overexpression in the bone microenvironment causes a transient decrease in trabecular volume and osteopenia due to impaired osteoblastic function.

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