

## Osteoblast-specific expression of insulin-like growth factor-1 in bone of transgenic mice induces insulin-like growth factor binding protein-5

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### Abstract

The activities of insulin-like growth factors (IGFs) in bone are modulated by a family of binding proteins (IGFBPs) whose physiological roles remain poorly understood. We have previously shown that targeted overexpression of IGF-I in osteoblasts of transgenic (OC-IGF-I) mice stimulates bone formation. In this model, bone formation is markedly but transiently increased in an age-dependent manner, raising the possibility that IGF-I may be influencing IGFBPs to in turn modulate its paracrine actions within bone. We sought to characterize the IGFBPs in normal mouse bone during development and to determine whether osteoblast-targeted overexpression of IGF-I influenced bone IGFBP abundance in vivo. Femoral bone IGFBP content was assessed in control nontransgenic and OC-IGF-I mice by I<sup>125</sup>-IGF-I ligand and immunoblotting. Bone IGFBP-5 and IGF-I mRNA abundance was determined using real-time reverse transcription (RT)-PCR. Ligand blot of bone extract showed a 30-kDa band, identified as IGFBP-5 by immunoblot, predominated. The abundance of IGFBP-5 declined with age in both control and transgenic bone. Ligand and immunoblot analysis revealed a 5-fold increase in IGFBP-5 protein levels at 3 weeks in transgenic bone ( $P < 0.0001$ ). The elevated IGFBP-5 protein levels were associated with a similar increase in IGF-I mRNA abundance (4-fold,  $P < 0.01$ ) and a significant increase in IGFBP-5 mRNA abundance (1.5-fold). Despite the age-related decline at 6 weeks, IGFBP-5 remained significantly ( $P < 0.01$ ) more abundant in transgenic bone compared to controls. In contrast, bone IGFBP-4 abundance was relatively unchanged by either age or IGF-I overexpression. These studies demonstrate a distinctive developmental pattern of IGFBP-5 content in mouse bone and show that osteoblast-derived IGF-I determines skeletal IGFBP-5 abundance, at least in part by inducing its synthesis. In that IGFBP-5 is thought to stimulate bone formation, directly or via IGF-I action, such changes in bone IGFBP-5 may be important to ensure robust bone acquisition in the early postnatal period.

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### Introduction

Insulin-like growth factors (IGFs) exert profound effects on somatic growth and cellular proliferation of many tissues and play an essential role in bone metabolism [1–3]. They have been implicated as regulators of osteoblast abundance,

having both proliferative and anti-apoptotic properties, and they accelerate osteoblast differentiation [4–7]. They also appear to help maintain bone matrix by enhancing osteoblastic matrix production [8,9] and by regulating osteoclastic bone resorption [10]. Tight control of these important IGF-mediated actions is achieved by a complex system operating within the bone that involves regulation of IGF production, interactions with specific binding proteins (IGFBPs), and activity of proteases that cleave the IGFBPs [4].

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The IGFbps are a family of six structurally homologous proteins that bind IGFs with high affinity and are thought to modulate the bioavailability of the IGFs within tissues [11,12]. IGFbps-4 and -5 are the most abundant IGFbps of bone [4,5,13–15]. Although the physiological roles of IGFBP-5 remain incompletely characterized, it is bound to hydroxyapatite within bone matrix [15–17], where it appears to sequester IGFs. IGFBP-5 is thought to enhance the skeletal actions of the IGFs [15,18–22], although under certain experimental conditions it may have inhibitory effects on bone [23–25]. In contrast, most studies suggest that IGFBP-4 generally antagonizes the anabolic effects of IGF-I on bone cells [14,19,26,27].

Much of our knowledge of the function and regulation of IGFbps in bone is derived from *in vitro* experiments, wherein the three-dimensional architecture and interactions among cell types are lost. Our laboratory has created mice with selective genetic alterations in individual components of the IGF regulatory system in order to study the actions of locally produced IGF-I in its native skeletal environment. These manipulations profoundly influence bone cell function and consequently bone formation and mineralization. In this regard, mice with targeted overexpression of IGF-I in osteoblasts (OC-IGF-I mice) exhibit markedly increased bone formation and increased trabecular and cortical bone volume in an age-dependent manner [28]. Because of the putative roles for the IGFbps as modulators of bone cell IGF exposure and the dynamic changes in bone formation observed during development and with changes in IGF-I expression, we examined the pattern and abundance of IGFbps in normal mouse bone during development, as well as that exposed to higher levels of IGF-I produced locally via transgenic expression. Our results indicate that bone IGFBP-5 is particularly abundant in young mice and that paracrine expression of IGF-I increases the production of bone IGFBP-5, findings that support a partnership between IGFs and IGFBP-5 in the promotion of bone mineralization.

## Materials and methods

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

FVB-N mice carrying an osteocalcin promoter-driven IGF-I transgene [28] and nontransgenic littermates were used for these studies. Two lines of transgenic mice were previously studied extensively, both of which manifested anabolic effects of paracrine IGF-I on bone [28]. We selected the line with the more robust transgene expression (line 36) to further define the effects of paracrine IGF-I on bone IGFBP content. These OC-IGF-I mice showed robust, osteoblast-restricted overexpression of IGF-I, with constant bone IGF-I mRNA levels from 3 to 24 weeks of age, and no changes in somatic growth or serum IGF-I concentrations compared to controls. Wild-type FVB-N mice ranging from

ages 2 to 12 weeks and OC-IGF-I mice aged 3 and 6 weeks were used to study developmental changes in bone IGFBP abundance. Animals of ages 3 and 6 weeks were also chosen to study the effects of locally produced IGF-I on bone IGFbps in order to relate findings to the anabolic effects seen in OC-IGF-I mice of these ages [28]. All animals received humane care in compliance with the local Institutional Animal Care and Use Committee.

### *Bone protein extraction and quantitation*

Animals were sacrificed using carbon dioxide at ages dictated by experimental design. Whole femurs were carefully dissected to remove soft tissue, immediately placed in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . At the time of extraction, paired femurs were weighed and then pulverized using a mortar and pestle under liquid nitrogen. Bone protein was extracted with TRIZOL reagent (Life Technologies, Rockville, MD) using a modification of the manufacturer's protocol. The pulverized bone was suspended in 4 ml TRIZOL reagent and homogenized for 1 min with a power homogenizer (Tekmar, Cincinnati, OH) at 85 rpm. Following 5-min incubation at room temperature, the samples were treated with 0.8 ml chloroform and centrifuged at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The organic phase was removed and incubated in 1.2 ml 100% ethanol for 3 min at room temperature followed by centrifugation at  $2000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . Protein was precipitated from the supernatant by adding 6 ml isopropyl alcohol for 10 min at room temperature and sedimentation at  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatant was removed, and the protein pellet was washed sequentially with 8 ml 0.3 M guanidine hydrochloride in 95% ethanol for 20 min at room temperature, followed by centrifugation at  $7500 \times g$  for 5 min at  $4^{\circ}\text{C}$ , according to the manufacturer's protocol. The protein pellet was vortexed in 1.8 ml ethanol, incubated for a further 20 min at room temperature, and again centrifuged at  $7500 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The pellet was vacuum dried (over 5 to 10 min) and resuspended in 1% SDS with incubation at  $50^{\circ}\text{C}$ . Remaining insoluble material was removed by centrifugation, and the protein supernatant was stored at  $-70^{\circ}\text{C}$  for subsequent quantitation and experiments.

Protein content of the bone extracts was quantified using a micro-Bicinchoninic acid protein assay reagent kit (Pierce Chemical Company, Rockford, IL) [29] following the manufacturer's instructions. Bone protein concentrations were determined by comparison to a bovine serum albumin standard.

### *Radioligand and immunoblotting techniques*

IGFBPs from mouse bone protein extracts were characterized by ligand blotting using radiolabeled IGF-I and Western immunoblotting using antisera to IGFBP-5 [30]. Pilot studies confirmed that intact IGFbps-4 and -5 tolerated the extraction procedure and were recovered with equal

efficiency. However, lower molecular weight proteolytic fragments produced by experimental cleavage of IGFBP-5 were not efficiently recovered (data not shown). For the ligand blots, 100 µg of the bone protein samples was suspended in Laemmli solution under nonreduced conditions, boiled for 5 min, and then separated along with prestained molecular weight markers on 12% SDS-polyacrylamide gels. Proteins were electrotransferred onto nitrocellulose membranes and then incubated with 400,000 cpm  $^{125}$ I-IGF-I in buffer in a sealed bag at room temperature overnight on an aliquot mixer. Following washing, blots were subjected to phosphor-imaging (STORM 820 PhosphorImager, Molecular Dynamics, Sunnyvale, CA). IGFBP relative abundance was quantitated using ImageQuant software (Molecular Dynamics) and analyzed by two-tailed Student's *t* tests ( $P < 0.05$  considered statistically significant).

For immunoblotting experiments, 150-µg bone protein samples were prepared under reducing conditions, but otherwise subjected to SDS-PAGE and electrotransferred onto nitrocellulose membranes as described. After 30 min exposure to 4% nonfat dried milk in TBST buffer (pH 7.4, 0.4 M Tris, 0.6 M sodium chloride, 0.05% Tween, 0.1% thimerosol), membranes were incubated with antisera to IGFBP-5 at a 1:100 dilution overnight. Experiments were repeated using two different IGFBP-5 primary antibodies in order to confirm findings and attempt to identify potential IGFBP-5 fragments. Experiments were initially performed using an IGFBP-5 antibody that recognizes murine IGFBP-5 and was raised in a goat against a carboxy terminal peptide of human IGFBP-5 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Experiments were later repeated using a mouse IGFBP-5-specific goat IgG, raised against recombinant mouse IGFBP-5 (R&D Systems, Inc., Minneapolis, MN), and shown to recognize the partially proteolyzed forms of IGFBP-5 typical in MC3T3 osteoblast conditioned media (data not shown). After incubation with the primary antibody, membranes were washed and incubated with secondary donkey anti-goat horseradish peroxidase-conjugated whole antibody (CHEMICON International, Inc., Temecula, CA) at a 1:5000 dilution in TBST/4% nonfat dried milk for 1 h, then further washed. IGFBP-5 antigen-antibody complexes were identified using enhanced chemiluminescence detection reagents (PICO and FEMTO, Pierce Chemical Company), followed by autoradiography.

#### Real-time RT-PCR

Total RNA was purified using STAT-60 reagent according to the manufacturer's instructions (Tel-Test, Inc., Friendswood, TX) and stored at  $-80^{\circ}\text{C}$  until use. For reverse transcription (RT)-PCR, 2–5 µg of total RNA was used for preparation of complementary DNA (cDNA) using a superscript kit (Invitrogen). The RT reaction mixture (20 µl) consisted of 5× first strand buffer, 0.5 mM dNTP, 50 nM oligo(dT) primers, and 20 U Superscript II reverse tran-

scriptase. Quantitative, real-time PCR was performed using the SYBR Green method on the Smart Cycler System (Cepheid, Sunnydale, CA). Each 25 µl of SYBR Green reaction mixture consisted of 1 µl of cDNA, 12.5 µl of 2× Universal SYBR Green PCR master mix (Qiagen, Valencia, CA), and 0.5 µM of forward and reverse primers. Primers generating products of approximately 100–120 bps were designed to corresponding flanking regions of exons 1 and 2 of the mouse IGFBP-5 and exons 3 and 4 of the mouse  $\beta$ -actin gene using the Gene Runner program and PCR. The forward primer for IGFBP-5 was 5'-TTTG-CCTCAACGAAAAGAGC-3' and the reverse primer was 5'-GTAGGTCTCTTCAGCCATCTCG-3'. The forward primer for IGF-I was 5'-CACTCATCCACAATGC-CTGTCT-3' and the reverse primer was 5'-CTGAGCTG-GTGGATGCTCTTC-3' [31]. The forward primer for  $\beta$ -actin was 5'-CTGAACCCCTAAGGCCAACCGTG 3' and the reverse primer was 5'-GGCATAACAGGGACAGCA-CAGCC-3'. Predicted cycle thresholds were calculated using Smart Cycler software and a melting curve was produced by slow denaturation of the PCR products to validate the specificity of amplification. Data were analyzed using the Q-Gen software for quantitative real-time PCR [32]. Each sample was tested in triplicate with quantitative PCR. Three samples obtained from each group of wild type and OC-IGF-I mice for IGFBP-5 mRNA experiments, and four to six samples from each group for IGF-I mRNA experiments, were used to calculate the means and standard errors (SE). Data were analyzed using two-tailed Student's *t* tests and two-tailed Mann-Whitney rank sum tests.

## Results

### Characterization of IGFBPs from normal mouse bone

The forms and abundance of IGFBPs found in normal mouse bone were examined by ligand blot (Fig. 1). Of the three major bands observed, the most abundant was a 30-kDa band that co-migrated with the IGFBP-5 control. This band, identified by immunoblot as IGFBP-5, was flanked by fainter bands representing variably glycosylated forms of the binding protein. IGFBP-5 associated with extracellular matrix typically exists in the intact form [15,33–35], as was found in these experiments.

The fastest migrating band (24 kDa) was identified as IGFBP-4 by its characteristic molecular weight and position, and co-migration of an IGFBP-4 control. A 40-kDa band of lesser intensity was also observed and likely represents IGFBP-3 due to its apparent molecular weight and diffuseness suggesting variable degrees of glycosylation.

### Bone IGFBP-5 decreases with age

Developmental changes in bone IGFBP abundance in normal and OC-IGF-I mice were assessed by subjecting

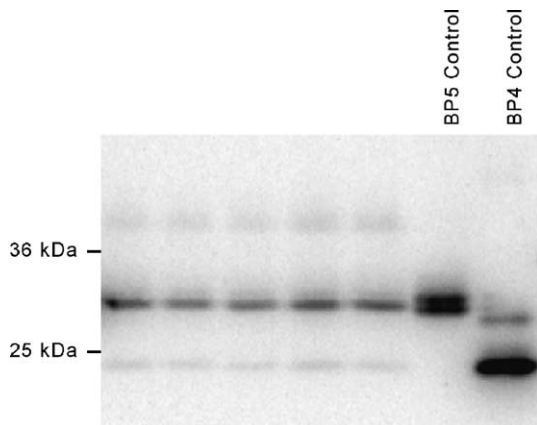


Fig. 1. IGFFBPs found in normal mouse bone. Representative  $^{125}\text{I}$ -IGF-I radioligand blot showing bone IGFBP-5 (30 kDa), IGFBP-4 (24 kDa), and IGFBP-3 (40 kDa). Each lane contained 100  $\mu\text{g}$  of femoral protein extract from a single mouse. IGFBP-5 (75 ng) and IGFBP-4 (50 ng) were included as controls. Molecular weight marker positions are shown on the left.

bone extracts from mice of increasing ages to immunoblotting and ligand blot analysis. Immunoreactive IGFBP-5 declined markedly and steadily from 2 to 12 weeks of age in normal mice (Figs. 2A and B). A similar age-related decline was observed by comparing 3- and 6-week-old OC-IGF-I transgenic mice (Fig. 2C). Only intact IGFBP-5 (30 kDa) was observed with the use of either IGFBP-5 antibody. Results were identical for both males and females (data for females not shown).

#### Bone IGFBP-5 is increased in OC-IGF-I mice

To determine the effect of osteoblast-derived IGF-I on bone IGFBP-5 abundance, comparisons were made in femurs from OC-IGF-I transgenic and control mice at 3 and 6 weeks of age.  $^{125}\text{I}$ -IGF-I radioligand blotting of bone protein extracted from 3-week-old mice showed that the 30-kDa band (shown to contain IGFBP-5) was increased 5-fold ( $P < 0.0001$ ) in male OC-IGF-I transgenic bone compared with nontransgenic littermate controls (Figs. 3A and E). A similar 5-fold increase ( $P = 0.001$ ) in IGFBP-5 abundance was observed in 3-week-old female transgenic mice relative to controls. By 6 weeks, the difference was still apparent, but was less pronounced at 1.5-fold ( $P < 0.01$ ) in male OC-IGF-I transgenic versus control bones (Figs. 3B and E), and 2.2-fold ( $P < 0.001$ ) in female transgenic bones compared with controls. Increases in immunoreactive IGFBP-5 observed using IGFBP-5 antisera mirrored those on ligand (Figs. 3C and D). Identical results were obtained using two different IGFBP-5 antibodies.

#### Effects of age and IGF-I on bone IGFBP-4

The abundance of IGFBP-4 declined only slightly with age in male mice, with a 1.25-fold relative abundance in 3-week-old compared with 6-week-old mice ( $P < 0.05$ ), and a 1.4-fold relative abundance in 3-week-old compared with

12-week-old mice ( $P < 0.05$ ), when assessed by  $^{125}\text{I}$ -IGF-I radioligand blot. No age-related difference in IGFBP-4 was detected in female mice. In further contrast to IGFBP-5, no change in bone IGFBP-4 abundance in the presence of osteoblast overexpression of IGF-I was detected in either age group in male and female mice (Figs. 3A, B, and E).

#### Bone IGFBP-5 mRNA abundance is increased by IGF-I at 3 weeks

To determine whether the increase in IGFBP-5 protein seen in 3-week-old OC-IGF-I transgenic bone might reflect increased IGFBP-5 messenger RNA (mRNA) expression, bone IGFBP-5 mRNA was quantitated by real-time RT-PCR. IGFBP-5 mRNA expression was significantly greater in OC-IGF-I transgenic bone compared to controls at 3 weeks ( $P < 0.05$ ) (Fig. 4A). Levels declined at 6 weeks in transgenic mice. In contrast, there was no significant change in IGFBP-5 mRNA expression in control mice at either age.

Evidence for posttranscriptional regulation of bone IGFBP-5 was also sought by attempted fragment detection. However, the recovery of IGFBP-5 fragments proved to be considerably less than that of intact IGFBP-5. Thus, we cannot exclude the involvement of proteolysis in the regulation of these binding proteins in vivo.

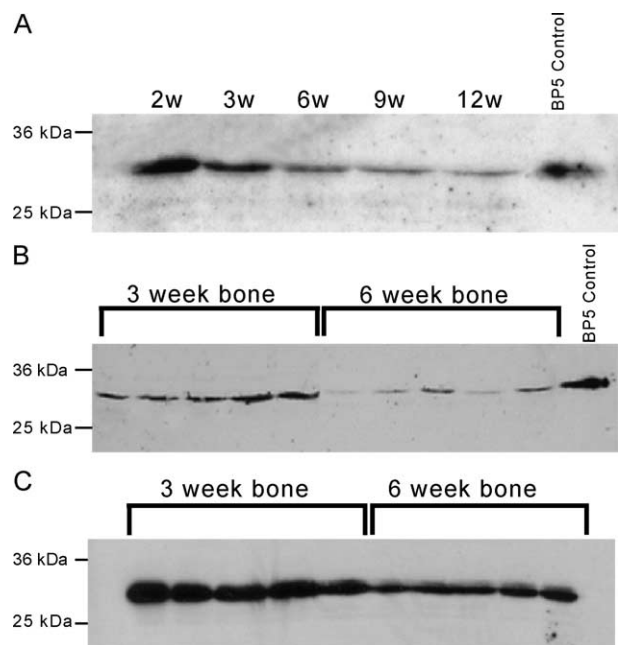


Fig. 2. Developmental changes in bone IGFBP-5 abundance in normal wild-type (A and B) and OC-IGF-I (C) male mice. Immunoblots were performed using a goat antihuman IGFBP-5 antibody (Santa Cruz Biotechnology, Inc.), as described in Materials and methods. (A) IGFBP-5 immunoblot showing declining abundance from 2 to 12 weeks (w) of age in normal mice. Each lane contained 150  $\mu\text{g}$  pooled femoral protein extract from five mice of the relevant age. (B) IGFBP-5 immunoblot showing decreasing levels from 3 to 6 weeks of age in normal mice. Each lane contained 150  $\mu\text{g}$  of bone protein extract from paired femurs of an individual animal. (C) IGFBP-5 immunoblot showing decreasing levels from 3 to 6 weeks of age in OC-IGF-I mice. Protein loaded as for B.

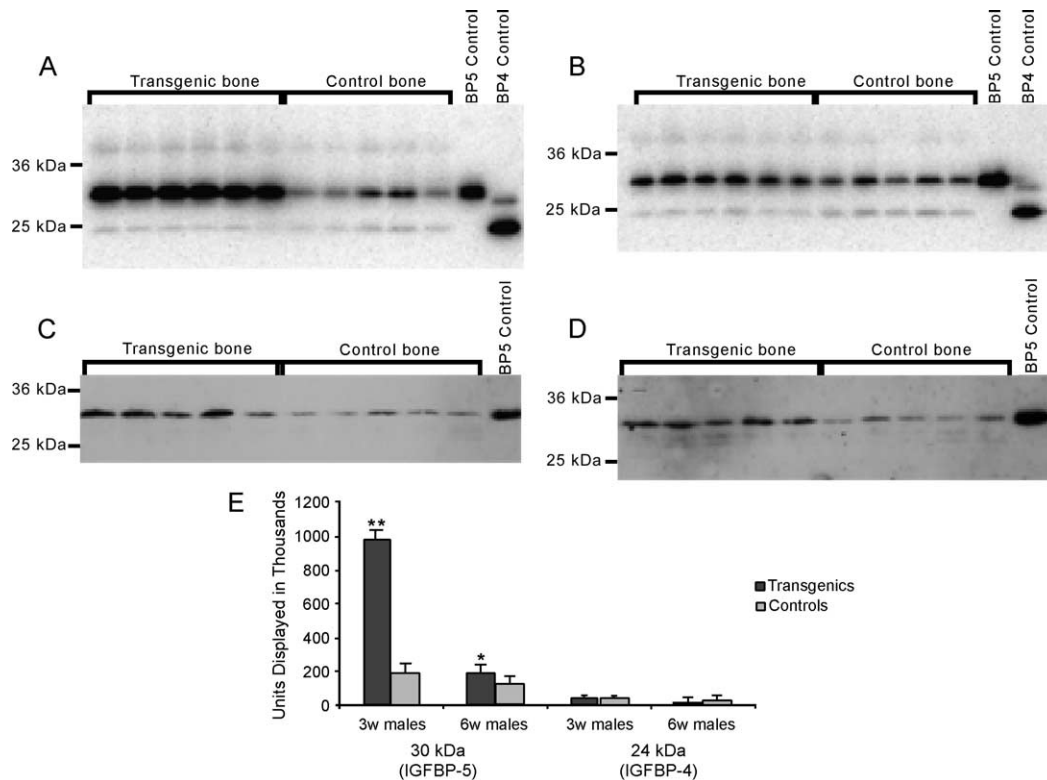


Fig. 3. Effect of osteoblast-derived IGF-I on bone IGFBP abundance.  $^{125}\text{I}$ -IGF-I radioligand and immunoblotting experiments comparing OC-IGF-I transgenic and nontransgenic control male mouse femoral bone. For radioligand blots, 100  $\mu\text{g}$  of extracted bone protein from individual mice was loaded into each lane. For immunoblots, 150  $\mu\text{g}$  of extracted bone protein was used. Immunoblots were performed using a goat antihuman IGFBP-5 antibody (Santa Cruz Biotechnology, Inc.). IGFBP-5 (75 ng) and IGFBP-4 (50 ng) were included as controls. Molecular weight marker positions are shown on the left. (A)  $^{125}\text{I}$ -IGF-I radioligand blot comparing OC-IGF-I transgenic and control mouse bone IGFBPs at 3 weeks of age. (B)  $^{125}\text{I}$ -IGF-I radioligand blot comparing OC-IGF-I transgenic and control mouse bone IGFBPs at 6 weeks of age. (C) IGFBP-5 immunoblot showing increased bone IGFBP-5 abundance in OC-IGF-I transgenic compared with control mice at 3 weeks of age. (D) IGFBP-5 immunoblot showing increased bone IGFBP-5 abundance in OC-IGF-I transgenic compared with control mice at 6 weeks of age. (E) Bone IGFBP-5 and -4 abundance in transgenic and control mice at ages 3 and 6 weeks: graphical data derived from  $^{125}\text{I}$ -IGF-I radioligand blots in A and B. IGFBP abundance is represented by phosphor-imaging units, displayed in thousands (mean  $\pm$  standard deviation). Asterisks indicate significant differences relative to controls:  $^{**}P < 0.0001$ ,  $^{*}P < 0.01$ .

#### OC-IGF-I mice have a sustained increase in bone IGF-I mRNA abundance

To further examine the relationship between IGF-I and IGFBP-5, bone IGF-I mRNA expression was also evaluated by real-time RT-PCR at 3 and 6 weeks of age. Bone IGF-I mRNA abundance was markedly increased in OC-IGF-I transgenic mice compared to age-matched wild-type littermates in the order of 4-fold ( $P < 0.01$ ) at 3 weeks and 3-fold ( $P < 0.05$ ) at 6 weeks of age (Fig. 4B). There was no statistically significant developmental change in IGF-I mRNA expression from 3 to 6 weeks in either OC-IGF-I mice or wild-type controls (Fig. 4B).

#### Discussion

Mice have become valuable experimental models of bone pathobiology because the principal bone trophic factors are represented and specific genes can be manipulated in vivo. Our study supports the applicability of studies of the IGF/

IGFBP axis in the murine system to man by showing that IGFBP-5 is the principal IGFBP of mouse bone as it is in human bone [15,17]. We now provide direct evidence that bone IGFBP-5 is increased by paracrine expression of IGF-I by osteoblasts. This finding indicates that the increase in skeletal IGFBP-5 abundance in humans with growth hormone excess [36] is likely mediated by local IGF-I, and suggests that IGFBP-5 is integrated into the physiologic actions of the IGFs in bone.

We also describe an age-related reduction in the abundance of bone IGFBP-5 protein, in further accord with studies in humans [17]. This normal developmental decline in bone IGFBP-5 could theoretically reflect a concurrent reduction in IGF-I exposure, but circulating IGF-I concentrations increase over the ages examined [37], restoration of bone IGF-I mRNA expression by transgenic means in our experiments does not obviate the decline, and IGF-I mRNA abundance does not decline between 3 and 6 weeks in normal wild-type mice. Therefore, the age-related decrease in mouse bone IGFBP-5 appears to occur independently of IGF-I.

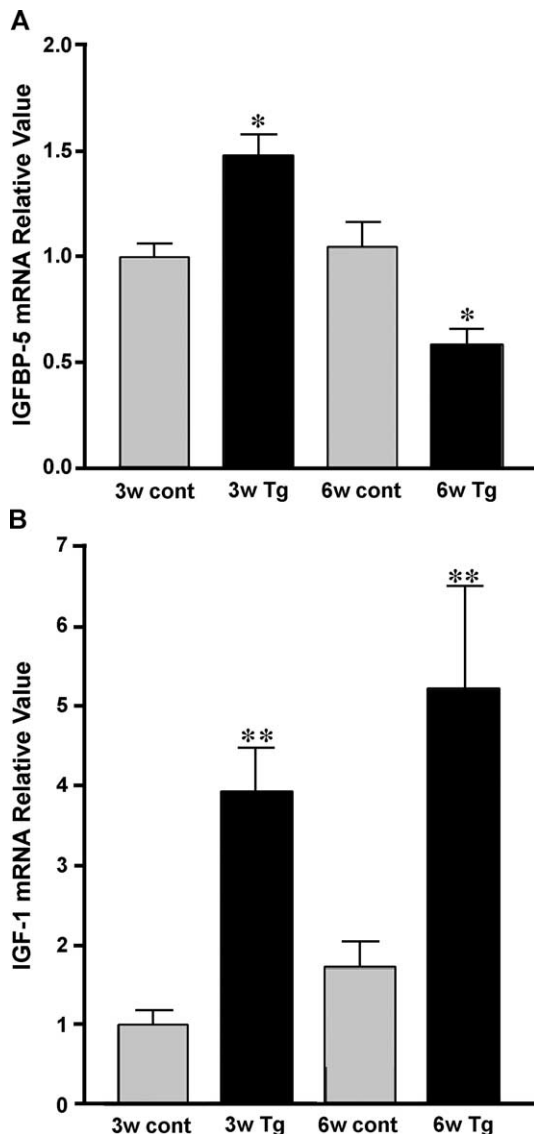


Fig. 4. IGFBP-5 and IGF-I mRNA abundance in bone from OC-IGF-I mice and controls. Total RNA was extracted from whole femurs from control nontransgenic (cont) and OC-IGF-I transgenic (Tg) mice as described in Materials and methods. mRNA levels were determined using real-time PCR and are expressed as fold difference (mean  $\pm$  SE) relative to 3-week-old control mice. The asterisks indicate a significant difference relative to 3-week controls (\* $P$  < 0.05, \*\* $P$  < 0.01). (A) IGFBP-5 mRNA relative abundance in transgenic and control mice at 3 and 6 weeks. (B) IGF-I mRNA abundance in transgenic and control mice at 3 and 6 weeks.

The mechanisms underlying the regulation of IGFBP-5 in mouse bone matrix may involve altered gene transcription and/or posttranslational events. We found that overexpression of IGF-I yielded a significant rise in IGFBP-5 mRNA in 3-week-old mice, implying that increased production explains at least some of the concurrent increment in bone IGFBP-5. In support of this mechanism are other works that include *in vitro* studies that show IGF-I promotes IGFBP-5 mRNA transcription in skeletal cells [34,38,39] and an *in vivo* report in which transgenic overexpression of IGF-I in mouse brain increased IGFBP-5 mRNA and protein abundance [40].

The abundance and properties of IGFBP-5 are also influenced by posttranslational mechanisms, principally involving proteolytic processing. Studies by Thrailkill et al. [41,42] showed that IGFBP-5 protein levels in cultured murine osteoblast (MC3T3) media peaked between days 8 and 14, coincident with the onset of osteoblast differentiation, but declined thereafter in association with increased IGFBP-5 proteolysis. Exposure to IGF peptides raises intact IGFBP-5 concentrations in bone cell and human fibroblast culture media by inhibiting protease-mediated degradation, implying a relative insensitivity of the IGFBP-5/IGF-I complex to proteolysis [23,43–45]. In our studies, variation in IGFBP-5 protease activity is a potential explanation for the age-related decline of IGFBP-5 in normal mice, since there was no concurrent change in IGFBP-5 mRNA. In addition, the increment in IGFBP-5 mRNA in response to transgenic overexpression of IGF-I in 3-week-old mice was modest (1.5-fold) compared to the robust (5-fold) change in protein abundance, which mirrored the 4-fold increase in IGF-I mRNA in OC-IGF-I transgenic bone. This suggests the changes in bone IGFBP-5 content also reflect protection from degradation by IGF-I. Although we sought evidence for proteolysis in the form of IGFBP-5 proteolytic fragments, none were detected in our bone extracts, despite the use of two specific IGFBP-5 antibodies recognizing both the C-terminus and whole molecule IGFBP-5. However, the extraction method employed does not appear to be well suited for detecting smaller IGFBP-5 fragments, and thus a significant role of proteolysis cannot be excluded.

The age-related decline in bone IGFBP-5 protein content in the OC-IGF-I transgenic mice was associated with a decrease in IGFBP-5 mRNA abundance, although interestingly below that of littermate controls at 6 weeks. While a decline in osteoblast number may have contributed [28], it is conceivable that overproduction of IGF-I by osteoblasts accelerates what would be an otherwise later physiological decline of IGFBP-5 mRNA expression, with corresponding changes in protein abundance lagging behind. In this regard, dynamic indices of bone formation are increased in 3-week-old OC-IGF-I mice relative to controls (in accordance with parallel changes in IGFBP-5 protein and mRNA), whereas at 6 weeks, dynamic indices in the OC-IGF-I transgenic mice trend lower than wild-type levels, suggesting an earlier decline in osteoblast function in transgenics [28]. Detailed analysis of the different kinetics of IGFBP-5 protein and mRNA in transgenic and wild-type bone is beyond the scope of our study.

The specific effects of alterations in bone IGFBP-5 abundance are difficult to predict because the amount, form and location of IGFBP-5 in bone ultimately determine whether this binding protein facilitates or inhibits IGF-I action [5,46]. IGFBP-5 inhibits bone formation in mice when massively overexpressed by osteoblasts [25]. Conversely, a naturally occurring carboxy-truncated form of IGFBP-5 stimulates osteoblast mitogenesis and enhances

IGF action when added to cultured bone cells [22], and similar anabolic effects have been observed in vivo following systemic administration of IGFBP-5 [18,20]. Given the apparent dichotomy, it is worthwhile to consider the changes in IGFBP-5 in the context of the skeletal phenotypes of these transgenic and normal animals as reported previously [28]. Mice with osteoblast-specific overexpression of IGF-I at 3 weeks demonstrate an increase in bone formation without a change in osteoblast number, indicating that IGF-I is stimulating the function of individual osteoblasts. That IGFBP-5 mRNA and protein also increase suggests this too is a feature of enhanced osteoblast function, and that there is a role for IGFBP-5 in the regulation of bone formation by IGF-I. By 6 weeks, the effect of IGF-I on bone formation is no longer evident and bone IGFBP-5 protein and mRNA have declined substantially. Because matrix-bound IGFBP-5 potentiates cell growth responsiveness to IGF peptides in vitro [15,33], it is possible that the age-related decline of IGFBP-5 in bone may explain the diminished capacity of IGF-I to stimulate bone formation in the older animals.

The lack of significant changes in bone IGFBP-4 with either age or IGF-I exposure was surprising. IGF treatment of human osteoblast cells in vitro results in the loss of intact IGFBP-4 in conditioned media due to proteolytic cleavage [47–50], resulting in enhancement of IGF-I-stimulated cell growth [48], and IGFBP-4 protein and mRNA levels have been shown to increase during MC3T3 osteoblast development [41]. Changes in IGFBP-4 production might be less apparent given our reliance on bone extract and the fact that IGFBP-4 does not possess a high affinity for bone matrix [15,35]. However, using this identical method, we easily observed increased bone IGFBP-4 in mice with osteoblast-specific IGFBP-4 overexpression [27]. Therefore, we believe that if age or IGF-I exposure had produced meaningful changes in bone intact IGFBP-4, they should have been revealed.

This transgenic model has provided a unique opportunity to examine the autocrine-paracrine actions of IGF-I in bone in vivo. Our findings suggest that IGFBP-5 is important in the developmental aspects of bone formation. Some of the effects likely involve interactions of IGFBP-5 with IGF-I whereas others reflect direct actions of the binding protein. Recent studies make it clear that the anabolic properties of IGFBP-5 are not solely dependent on IGF-I, as they are observed when tissues from IGF-I null mice are exposed to IGFBP-5 [51]. Better definition of the function of the IGF/IGFBP axis in bone in vivo will require analysis of the skeletal consequences in mice with additional selective genetic modifications in individual components of the IGF/IGFBP system.

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