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Insulin-Like Growth Factor-Binding Protein-5 Inhibits Osteoblast Differentiation and Skeletal Growth by Blocking Insulin-Like Growth Factor Actions

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Signaling through the IGF-I receptor by locally synthesized IGF-I or IGF-II is critical for normal skeletal development and for bone remodeling and repair throughout the lifespan. In most tissues, IGF actions are modulated by IGF-binding proteins (IGFBPs). IGFBP-5 is the most abundant IGFBP in bone, and previous studies have suggested that it may either enhance or inhibit osteoblast differentiation in culture and may facilitate or block bone growth *in vivo*. To resolve these contradictory observations and discern the mechanisms of action of IGFBP-5 in bone, we studied its effects in differentiating osteoblasts and in primary bone cultures. Purified wild-type (WT) mouse IGFBP-5 or a recombinant adenovirus expressing IGFBP-5^{WT} each prevented osteogenic differentiation induced by the cytokine bone morphogenetic protein (BMP)-2 at its earliest stages without interfering with BMP-mediated signaling, whereas an analog with reduced IGF binding (N domain mutant) was ineffective. When added at later phases of bone cell maturation, IGFBP-5^{WT} but not IGFBP-5^{N} blocked mineralization, prevented longitudinal growth of mouse metatarsal bones in short-term primary culture, and inhibited their endochondral ossification. Because an IGF-I variant (R^3IGF-I) with diminished affinity for IGFBPs promoted full osteogenic differentiation in the presence of IGFBP-5^{WT}, our results show that IGFBP-5 interferes with IGF action in osteoblasts and provides a framework for discerning mechanisms of collaboration between signal transduction pathways activated by BMPs and IGFs in bone. (*Molecular Endocrinology* 22: 1238–1250, 2008)

BONE REMODELING, THE dynamic process that maintains bone integrity and mass throughout adult life, consists of two phases, resorption by osteoclasts and new bone formation by osteoblasts (1, 2), and is controlled by an interplay between local and systemically derived signals mediated by hormones, growth factors, and other molecules acting in concert with a hierarchical program of bone-specific transcription factors (3). Among growth factors with positive actions on bone formation are the IGFs, IGF-I and -II (4), closely related single-chain proteins that bind with high affinity to the IGF-I receptor, a transmembrane ligand-activated tyrosine protein kinase related to the insulin receptor (5). The importance of IGF action in bone has been demonstrated by a series of genetic manipulations in mice. Systemic IGF-I receptor deficiency led to retarded skeletal development and delayed ossification and many other severe defects that contributed to neonatal death (6), whereas targeted loss of the receptor in osteoblasts caused diminished bone mass and reduced mineral apposition rate (7). In contrast, overexpression of IGF-I in osteoblasts led to an enhanced rate of bone formation and increased mineralization (8).

IGF-I and IGF-II also bind to a family of six secreted IGF-binding proteins (IGFBPs), IGFBP-1 through 6. IGFBPs play modifying roles in IGF action by regulating both IGF half-life and access to the IGF-I receptor (9, 10), although these proteins have been shown to modulate other biological processes independent of their ability to bind IGFs (11). The predominant IGFBPs in bone are IGFBP-4 and IGFBP-5 (12, 13). Although IGFBP-4 primarily blocks IGF actions in bone and inhibits osteogenesis (14–16), the effects of IGFBP-5 are less certain, and both inhibitory and stimulatory effects on osteoblast proliferation and differentiation have been described (11, 17–19). IGFBP-5 is a 252-amino-acid secreted protein consisting of highly conserved, cysteine-rich, amino- and carboxyl-terminal domains and a more weakly conserved central segment (19, 20). The amino-terminal region encodes the primary IGF-binding site, although the carboxyl-terminal segment also is needed for high-affinity growth factor binding (21–23). The carboxyl-terminal portion

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Abbreviations: Ad-BMP-2, Adenovirus encoding BMP-2; AP, alkaline phosphatase; BMP, bone morphogenetic protein; Dox, doxycycline; IGFBP, IGF-binding protein; OM, osteogenic media; Ocn, osteocalcin; Osx, osterix; PI3, phosphatidylinositol-3; WT, wild type.

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also contains determinants that interact with extracellular matrix proteins (24–26). In the developing embryo, IGFBP-5 is synthesized during endochondral bone formation by differentiating chondroblasts and osteoblasts (27–29), and it accumulates in adult bone (29). Despite this information, evidence defining specific roles for IGFBP-5 in bone remains both contradictory and limited. Global knockout of IGFBP-5 in mice had minimal impact on bone or on whole-animal physiology (30), although its overexpression caused impaired mineralization (31, 32). In contrast, systemic administration of IGFBP-5 with equimolar concentrations of IGF-I led to increased cortical bone formation and enhanced bone mineral density in mice (33). Stimulatory effects of IGFBP-5 in combination with IGF-I also were observed after local injections into calvarial bone (34). Similarly contradictory results have been seen in cultured osteoblasts or in bone cell lines incubated with IGFBP-5, and both stimulatory and inhibitory outcomes have been reported (15, 35–38).

To address the roles of IGFBP-5 in bone, we purified mouse IGFBP-5 from a mammalian cell expression system, along with a modified version of the protein with reduced affinity for IGFs and studied effects of these molecules on osteoblast differentiation induced by the osteo-inductive growth factor, bone morphogenetic protein (BMP)-2 (39, 40), and on growth and mineralization of mouse metatarsal bones in primary culture. We find that native IGFBP-5 but not a mutant with diminished IGF binding inhibited all phases of BMP-2-induced osteoblast differentiation and function and also blocked metatarsal growth. Because R^3IGF-I, a variant with full potency for the IGF-I receptor but diminished binding to IGFBPs, restored normal osteogenic differentiation in the presence of IGFBP-5, our results indicate that IGF action is required for osteoblast differentiation and demonstrate that IGFBP-5 acts primarily as an inhibitor of IGF-mediated signaling in bone.

RESULTS

Rapid Osteoblast Commitment and Differentiation after Infection of Mesenchymal Stem Cells with a BMP-2 Adenovirus

To study regulation of osteoblast differentiation by IGF-mediated mechanisms, we treated cultured mesenchymal stem cells with BMP-2 by adenovirus-mediated gene transfer (Fig. 1A) and incubated cells in osteogenic medium, which contained 10% fetal calf serum plus ascorbic acid and β-glycerol phosphate to aid in mineralization (31, 37). Using purified recombinant adenoviruses encoding rat BMP-2 (Ad-BMP-2) at a multiplicity of infection of 500, we were able to detect BMP-2 in conditioned medium within 1 d, which was not seen in cells infected with a control virus, Ad-β-Gal (Fig. 1B). Accompanying the rapid accumulation of BMP-2 in culture medium was the progressive and

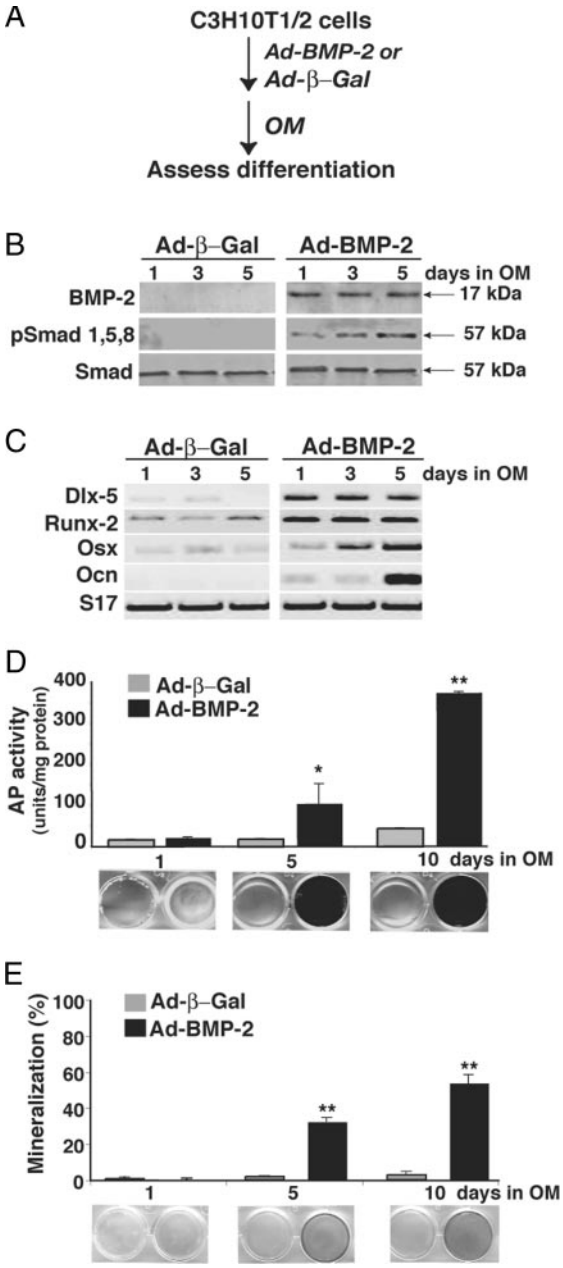

Fig. 1. BMP-2 Promotes Osteogenesis in C3H10T1/2 Cells

Results are shown of experiments in which C3H10T1/2 cells were infected with Ad-β-gal or Ad-BMP-2, followed by incubation in OM for up to 10 d as described in *Materials and Methods*. A, Experimental scheme; B, immunoblots of conditioned OM for BMP-2 and of whole-cell protein lysates for phosphorylated Smad (pSmad) and Smad after incubation of cells in OM for 1, 3, or 5 d; C, results of RT-PCR assays showing expression of osteoblast-specific genes Dlx-5, Runx-2, Osx, and Ocn and control gene S17 after incubation for 1, 3, or 5 d in OM; D, measurement of AP activity in lysates of cells incubated for 1, 5, or 10 d in OM (mean $\pm$ SD, n = 3; *, $P < 0.05$; **, $P < 0.0001$ *vs.* cells infected with Ad-β-gal). Representative images of qualitative AP staining at each time point are depicted *below* the graph. E, Mineralized area (mean $\pm$ SD, n = 5; **, $P < 0.01$ *vs.* Ad-β-gal-infected cells) was calculated as described in *Materials and Methods*. *Below* the graph are representative images at each time point of cells stained with Alizarin red to detect extracellular calcium deposition.

sustained induction of intracellular signaling through BMP receptors, as measured by phosphorylation of Smads 1, 5, and 8 in protein extracts, beginning by 1 d after viral infection (Fig. 1B), and the production of mRNAs encoding transcription factors Dlx-5 and Runx-2 (Fig. 1C), both known downstream targets of BMP-2-activated signaling pathways in bone (41–43). Accumulation of Dlx-5 and Runx-2 transcripts was followed temporally by the appearance of mRNAs encoding the transcription factor osterix (Osx) by d 3, and the bone-specific secreted protein osteocalcin (Ocn) by d 5 (Fig. 1C). Treatment of cells with Ad-BMP-2 also triggered the subsequent production of bone-specific alkaline phosphatase (AP) and accumulation of mineralized bone matrix, as measured, respectively, by AP enzymatic activity assays and Alizarin red staining, which were both detectable by d 5 and had increased by more than 20-fold over baseline at d 10 after adenoviral infection (Fig. 1, D and E). Taken together, these results demonstrate robust BMP-initiated osteoblast commitment and differentiation.

IGFBP-5 Inhibits BMP-2-Induced Osteoblast Differentiation in Mesenchymal Stem Cells by IGF-Dependent Mechanisms

IGFBP-5 is the most abundant IGFBP expressed in bone (11, 13, 29). Contradictory observations showing either stimulatory or inhibitory effects of this protein on osteoblast differentiation have been reported, and it has been suggested that the stimulatory actions may be IGF independent (11, 17–19). We performed a series of experiments to address these questions by examining the effects of addition of IGFBP-5 on BMP-2-mediated osteoblast differentiation (Fig. 2A). We used a mammalian overexpression system to prepare and subsequently purify both wild-type (WT) mouse IGFBP-5 (IGFBP-5^{WT}) and an amino acid substitution N-domain mutant with diminished IGF binding affinity (IGFBP-5^{N}), which we purified by heparin-affinity chromatography (44). Incubation of Ad-BMP-2-infected cells with these proteins led to their accumulation in conditioned medium at similar levels 48 h later, which was just before the medium change (the amount of IGFBP-5 declined to about 33% of starting values over this interval; (data not shown). As expected, IGFBP-5^{WT} could bind IGF-II as assessed by ligand blotting, whereas IGFBP-5^{N} could not (Fig. 2B). Neither IGFBP-5^{WT} nor IGFBP-5^{N} (150 nM) inhibited production or secretion of BMP-2, and neither reduced BMP-2-stimulated Smad phosphorylation (Fig. 2C). By contrast, IGFBP-5^{WT} blocked both tyrosine phosphorylation of the IGF-I receptor and serine phosphorylation of Akt, a signaling intermediate that is downstream of the activated IGF-I receptor, whereas IGFBP-5^{N} did not (Fig. 2C). Addition of IGFBP-5^{WT} prevented BMP-2-initiated expression of Dlx-5, Runx-2, Osx, and Ocn mRNAs (Fig. 2D) and completely inhibited induction of AP activity and bone matrix mineralization (Fig. 2, E and F). In contrast, incubation of cells with IGFBP-5^{N} did not impair any aspect of biochemical or morphological differentiation (Fig. 2, D and F). In addition, although other studies have shown that IGFBP-5 can promote osteoblast proliferation (15, 34, 45), neither IGFBP-5^{WT} nor IGFBP-5^{N} caused a change in the number of viable cells compared with controls (data not shown).

Several published studies have indicated that the mode of delivery of IGFBP-5 may influence its biological effects on cells, with overexpression leading to different outcomes than exogenous addition of the protein (46, 47). To address this issue, we examined results of delivery of IGFBP-5 by doxycycline (Dox)-inhibited recombinant adenoviruses on BMP-2-mediated osteoblast differentiation (see experimental scheme in Fig. 3A). Addition of Ad-IGFBP-5^{WT} prevented BMP-2-stimulated expression of Dlx-5, Osx, and Ocn mRNAs, reduced Runx-2 mRNA accumulation, eliminated induction of AP activity and bone matrix mineralization, and blocked a Runx2-dependent promoter-reporter gene (Fig. 3, B–E). These inhibitory actions of IGFBP-5^{WT} were reversed by treatment of cells with Dox. By contrast, infection with Ad-IGFBP-5^{N} did not block biochemical or morphological differentiation (Fig. 3, B–E). Thus, based on results depicted in Figs. 2 and 3, we conclude that IGFBP-5^{WT} prevents BMP-2-stimulated osteoblast differentiation irrespective of mode of delivery and appears to act downstream of BMP-2 receptors and Smad activation.

We further tested the hypothesis that the inhibitory effects of IGFBP-5 on osteoblast differentiation are IGF dependent by incubating IGFBP-5^{WT}-treated cells with R^3IGF-I (5 nM), an IGF-I analog with low affinity for IGFBPs but normal affinity for the IGF-I receptor (48). As illustrated in Fig. 4, addition of R^3IGF-I restored induction of Dlx-5, Osx, and Ocn mRNAs by BMP-2 in the presence of IGFBP-5^{WT} (Fig. 4A) and reversed the inhibition of AP activity and matrix mineralization (Fig. 4B). Treatment with R^3IGF-I also led to the reappearance of phosphorylated Akt (Fig. 4C). Taken together, the results in Figs. 2–4 demonstrate that IGFBP-5 can block BMP-2-mediated osteoblast differentiation at the earliest steps and support the idea that IGF-activated signaling pathways collaborate with BMP-2-stimulated pathways to promote osteoblast differentiation.

IGFBP-5 Inhibits All Phases of Osteoblast Differentiation and Function

Osteoblast differentiation is a complex, multistep process. After commitment of multipotent precursors to the osteoblast lineage and initiation of differentiation, manifested in part by expression of osteoblast-specific transcription factors (3, 41, 49), maturation proceeds with production of bone-specific proteins, deposition of extracellular matrix, and its subsequent mineralization (1, 50). As seen in Figs. 2–4, addition of IGFBP-5^{WT} to mesenchymal stem cells soon after onset of action of BMP-2 prevented osteoblast differentiation at its earliest stages. To begin to address how IGF-mediated signaling

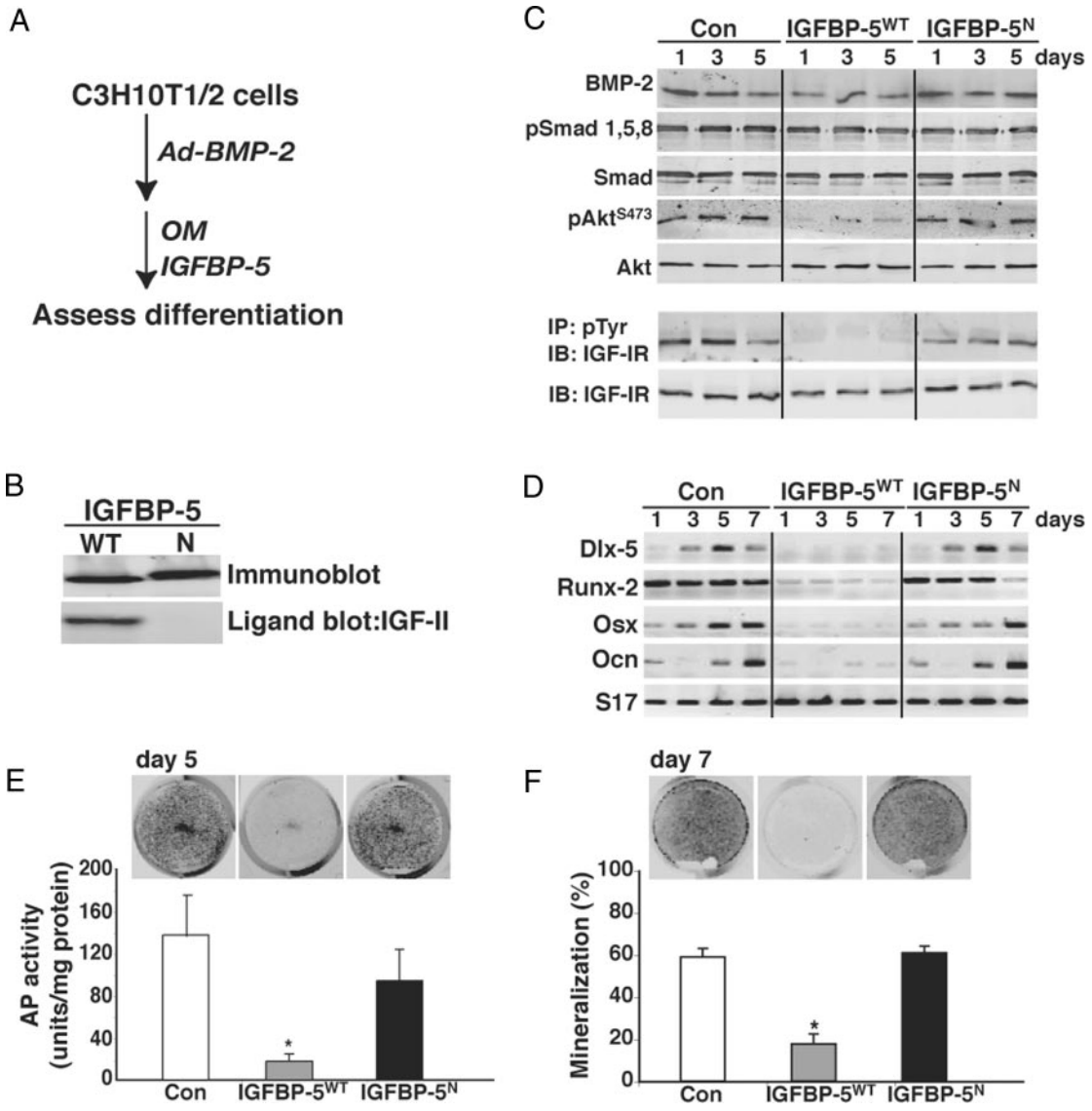

Fig. 2. IGFBP-5 Inhibits BMP-2-Induced Bone Differentiation

C3H10T1/2 cells were infected with Ad-BMP-2 and incubated in OM plus purified IGFBP-5 (150 nM) for up to 7 d. OM and IGFBP-5 were replaced every 48 h. A, Experimental scheme; B, detection of IGFBP-5^{WT} and IGFBP-5^{N} by immunoblotting (*upper panel*) and by IGF-II ligand blotting (*lower panel*) at 48 h after addition to OM; C, *upper panel*, detection of BMP-2 in conditioned OM and detection of phosphorylated Smad (pSmad 1, 5, and 8), Smad, Akt phosphorylated at serine 473 ($pAkt^{S473}$), and Akt in whole-cell protein lysates; *lower panel*, detection of tyrosine-phosphorylated IGF-I receptor β-subunit by immunoblotting after immunoprecipitation (IP) with anti-phosphotyrosine antibody and detection of IGF-I receptor β-subunit by immunoblotting (IB) of whole-cell protein lysates; D, results of RT-PCR assays showing expression of osteoblast-specific genes after incubation in OM for 5 d with IGFBP-5^{WT} or IGFBP-5^{N}; E, AP activity after 5 d in OM (mean ± SD, n = 3; *, $P < 0.01$ *vs.* control); F, mineralized area at d 7 assessed by Alizarin red staining (mean ± SD, n = 5; *, $P < 0.01$ *vs.* control).

pathways collaborate with BMP-2 to facilitate osteoblast differentiation, we incubated cells with IGFBP-5^{WT} starting at different times after infection with Ad-BMP-2 (see experimental scheme, Fig. 5A). As pictured in Fig. 5B, addition of IGFBP-5^{WT} at d 0 or 3 of differentiation prevented induction of AP activity, whereas incubation starting at d 5 blocked further increases in AP, and addition at d 7 had no effect. Analysis of osteoblast gene expression revealed complete inhibition by IGFBP-5^{WT} when added at d 0 or 3, substantial diminution at d 5, and smaller effects at d 7 (Fig. 5C, and data not shown). The most striking observation was lack of induction of Ocn mRNA when IGFBP-5^{WT} was added to cells as late as d 7, even though transcripts encoding factors known to regulate Ocn gene expression, including Runx-2 and Osx, were expressed at relatively normal levels (Fig. 5C). Incubation of cells at different time points with IGFBP-5^{WT} also consistently impaired subsequent matrix mineralization (Fig. 5D). Addition at d 5 or 7 prevented the normal approximately 50% increase in mineralization

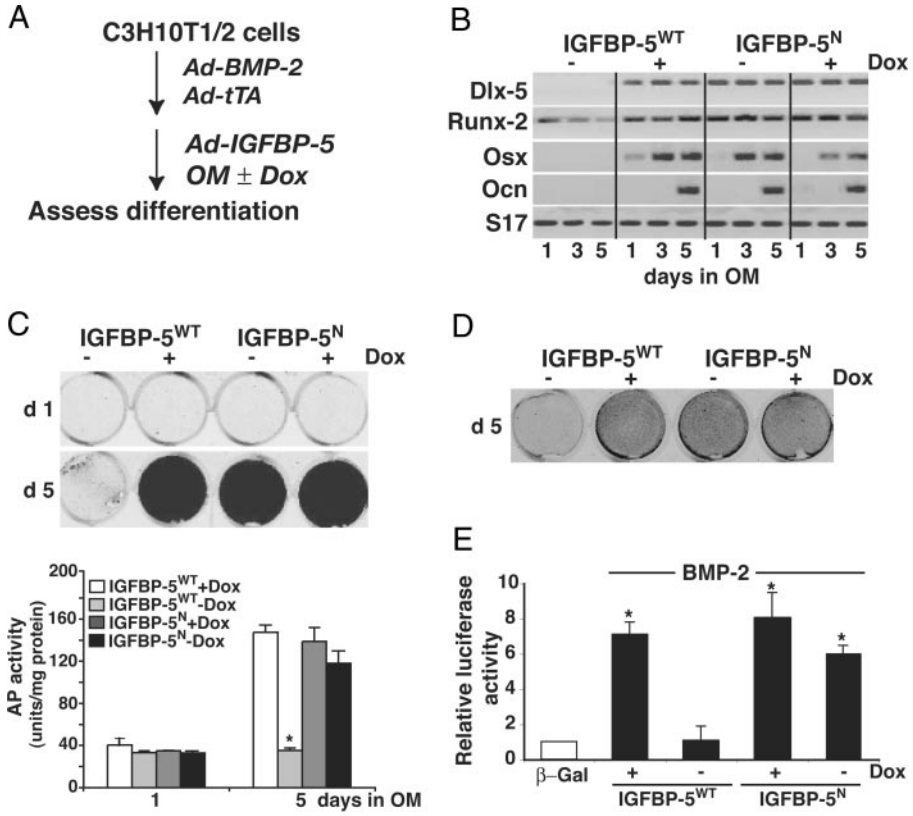

Fig. 3. Overexpression of IGFBP-5 Inhibits BMP-2-Mediated Osteoblast Differentiation

C3H10T1/2 cells were infected with Ad-BMP-2 and Ad-tTA followed 1 d later by infection with Ad-IGFBP-5^{WT} or a variant (Ad-IGFBP-5^{N}) with decreased binding of IGF-I or IGF-II and incubated in OM for up to 5 d. A, Experimental scheme; B, results of RT-PCR assays showing expression of osteoblast-specific genes after incubation in OM for 1, 3, or 5 d; C, detection of AP activity at d 1 and 5 by staining and by quantitative assay (mean ± SD, n = 3; *, $P < 0.0005$ *vs.* other groups at d 5); D, assessment of calcium deposition by Alizarin red staining after 5 d in OM; E, results of luciferase assays after transient transfection of C3H10T1/2 cells with a luciferase-reporter gene containing a minimal promoter plus six copies of the Ocn OSE-2 element. Transfected cells were infected with either Ad-β-gal or Ad-BMP-2 and Ad-tTA, followed by infection with Ad-IGFBP-5^{WT} or Ad-IGFBP-5^{N} and incubation in OM for 48 h (mean ± SD, n = 5; *, $P < 0.01$ *vs.* Ad-β-gal-infected cells).

that occurred during the next 5–7 d (Fig. 5D), even though at the latter time point, IGFBP-5^{WT} had no inhibitory effects on AP activity. Taken together, these results underscore key roles for IGF-mediated signal transduction pathways in all stages of *in vitro* osteoblast differentiation and maturation.

IGFBP-5 Inhibits Growth of Isolated Mouse Metatarsal Bones

Ex vivo culture of neonatal mouse metatarsal bones has been used to study bone growth and endochondral ossification (51–53). As shown in Fig. 6, these bones increased in length by about 33% in serum-free medium over a 10-d culture period, whereas a single addition of IGFBP-5^{WT} (200 nM) at d 1 reduced growth to about 5% over the same interval. In contrast, metatarsals incubated with IGFBP-5^{N} (200 nM) grew as robustly as control bones. The inhibitory effects of IGFBP-5^{WT} were dose dependent, with a single addition of as little as 50 nM causing an approximately 35% decline, and 200 nM reducing longitudinal growth by more than 70% compared with untreated controls (Fig. 7).

The mid-diaphysis region of metatarsal bones contains osteoblasts, osteoclasts, and hypertrophic chondrocytes and undergoes mineralization during *ex vivo* culture (51, 54), which can be measured by calcein staining. Under control conditions, there was an approximately 14% increase in the length of the mineralized zone over a 10-d incubation period, which was completely inhibited after a single addition of IGFBP-5^{WT} (200 nM) on d 1 but not by an equivalent dose of IGFBP-5^{N} (Fig. 8). Taken together, the results in Figs. 6–8 demonstrate that IGFBP-5^{WT} can block bone growth and mineralization in metatarsal bone cultures under serum-free conditions, although it remains to be determined which cell types would normally be the targets of IGF-mediated signaling pathways. Because exogenous growth factors were not added to the culture medium and because IGFBP-5^{N} was a completely ineffective inhibitor, our results additionally show that IGFs, produced by these metatarsals during *ex vivo*

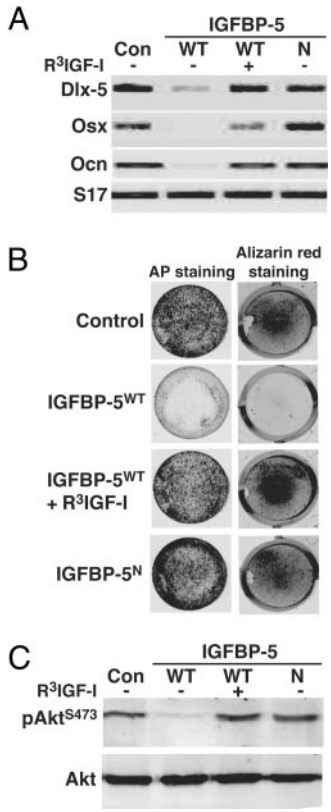

Fig. 4. IGF-I Reverses the Inhibitory Effects of IGFBP-5 on BMP-2-Induced Osteogenesis

Ad-BMP-2 infected C3H10T1/2 cells were incubated in OM plus purified IGFBP-5^{WT} or IGFBP-5^{N} (150 nM) with or without R^3IGF-I (5 nM). A, Results of RT-PCR assays showing expression of Dlx-5, Osx, Ocn, and S17 mRNAs at d 7; B, measurement of AP activity (AP staining) and mineralization (Alizarin red staining) at d 7; C, immunoblots of whole-cell protein lysates for Akt phosphorylated at serine 473 ($pAkt^{S473}$) and Akt at d 7.

incubation, are essential for both normal longitudinal growth and endochondral ossification.

DISCUSSION

Bone development and growth are tightly regulated processes modulated by multiple growth factors, including the IGFs. Through gene knockout experiments in mice and other studies, it has been established that IGF action plays a key role in normal skeletal development and bone remodeling (6–8). It is also known that IGFBPs regulate the activity of IGF-I and IGF-II (10). Of the six IGFBPs, IGFBP-5 is the most conserved and the most abundant in bone (13, 19). However, the role of IGFBP-5 in osteogenesis remains controversial, because contradictory studies have demonstrated both positive and negative effects on bone cell proliferation, osteoblast differentiation, and bone growth (17–19). In this report, we show that IGFBP-5 inhibits BMP-2-induced osteoblast differentiation and function and blocks longitudinal growth and mineralization of neonatal mouse metatarsal bones in short-term primary culture. Based on these results, we conclude that at least at the concentrations used in this study, IGFBP-5 primarily acts by inhibiting IGF actions in bone cells.

IGFBP-5 Inhibits Osteogenesis by Blocking IGF Actions

BMP-2 drives uncommitted mesenchymal progenitors toward the osteoblast lineage (40). As shown in Fig. 1, we were able to recapitulate this process in C3H10T1/2 fetal fibroblasts using adenovirus-mediated delivery of BMP-2, leading to rapid and robust induction of AP activity and extensive mineralization within several days. In this model of osteogenesis, either addition of purified mouse IGFBP-5 to osteogenic medium or delivery of IGFBP-5 by recombinant adenovirus was sufficient to block all phases of BMP-2-mediated osteoblast commitment and differentiation. In contrast, a modified IGFBP-5 containing amino acid substitutions that reduced its affinity for IGF-I and IGF-II (IGFBP-5^{N}) was inert (Figs. 2 and 3). The inhibitory effects of IGFBP-5 on osteoblast differentiation could be reversed by addition of an IGF-I variant with low affinity for IGFBPs and normal affinity for the IGF-I receptor (Fig. 4), further indicating that the effects of IGFBP-5 were dependent on its ability to block IGF actions. Taken together with results seen using short-term primary metatarsal cultures, in which IGFBP-5^{WT} but not IGFBP-5^{N} prevented both longitudinal bone growth and matrix mineralization (Figs. 6–8), we conclude not only that IGF action is required for BMP-2-stimulated osteoblast differentiation but also that IGFBP-5 acts primarily as an inhibitor of IGF-mediated signaling in bone. Studies performed with MC3T3 osteoblastic cells and with mouse osteosarcoma cell lines (31, 38) support our results, as do experiments demonstrating that IGFBP-5 transgenic mice have reduced bone density and defective mineralization (31, 32), but other published data are at odds with our conclusions. For example, addition of recombinant IGFBP-5 produced in *Escherichia coli* to osteosarcoma cells or to primary osteoblasts was shown to cause small increases in AP activity and Ocn expression (35), in contrast to the total inhibition that we observe. Perhaps the contradictory results can be explained by differences between *E. coli*-derived IGFBP-5 and the mammalian-generated IGFBP-5 used here.

Another consideration to explain the apparently diverse and discrepant actions of IGFBP-5 seen in previously published studies is its mode of delivery, which others have argued may modify its biological effects (46, 47). Although IGFBP-5 is a secreted protein, it has been postulated that its overexpression in cells enhances its intracellular accumulation, leading to subsequent actions in partnership with nuclear proteins

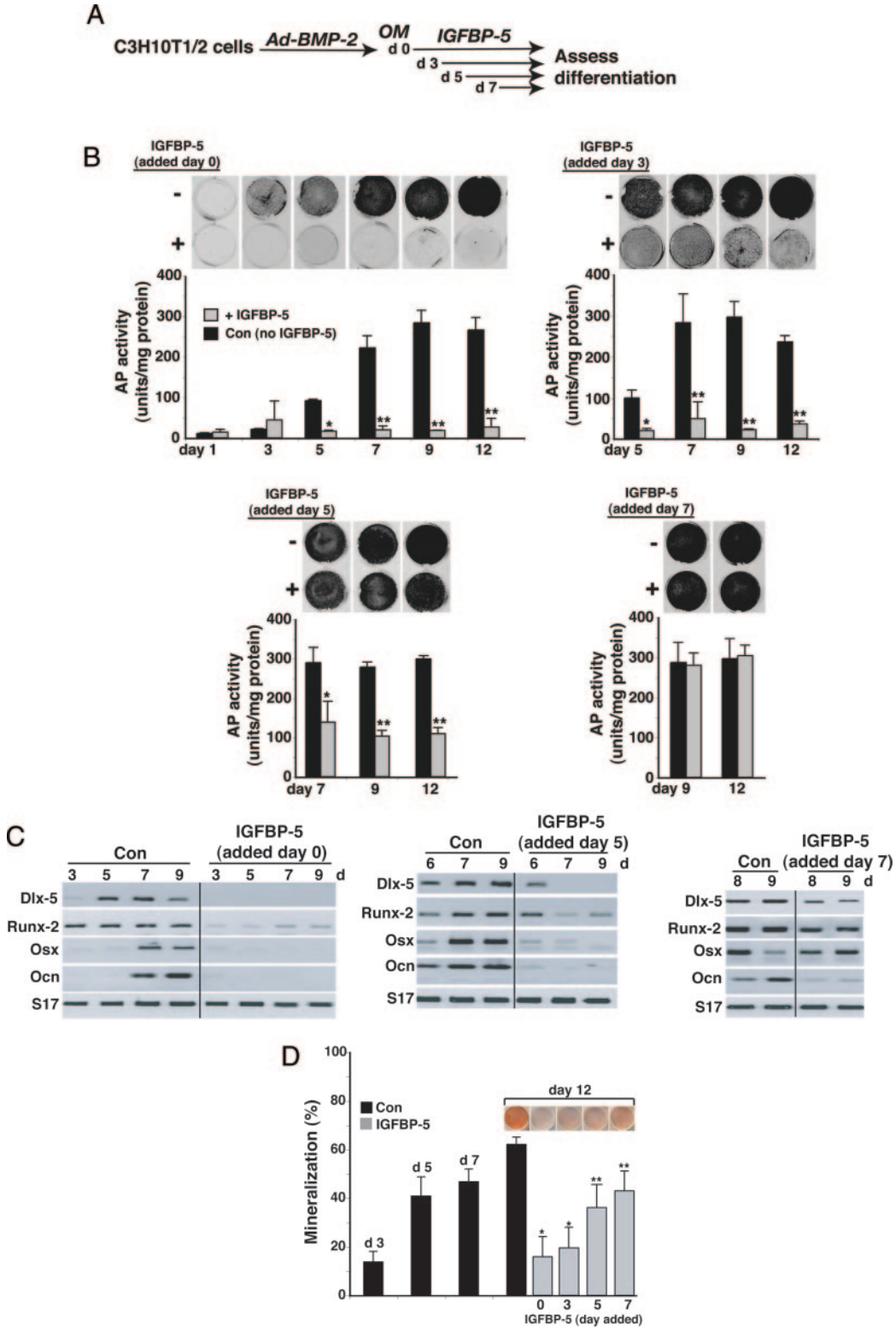

Fig. 5. IGFBP-5 Has Inhibitory Effects at Different Phases of Osteoblast Differentiation and Maturation

Ad-BMP-2-infected C3H10T1/2 cells were incubated in OM. Purified IGFBP-5^{WT} (150 nM) was added to the cells at d 0, 3, 5, or 7 and maintained in OM for up to 12 d. OM and IGFBP-5 were replaced every 48 h. A, Experimental scheme; B, AP staining and activity measured after addition of IGFBP-5^{WT} to OM at the indicated days [mean ± SD, n = 3; *, $P < 0.05$ *vs.* control (Con); **, $P < 0.007$ *vs.* Con]; C, results of RT-PCR assays showing expression of osteoblast-specific genes at different time points after addition of IGFBP-5^{WT}; D, mineralized area assessed at d 3, 5, 7, and 12 for control cells and at d 12 for cells incubated with IGFBP-5^{WT} for different durations (mean ± SD, n = 5; *, $P < 0.05$; **, $P < 0.01$ *vs.* control at d 12). Also shown are representative images of Alizarin *red*-stained cells at d 12.

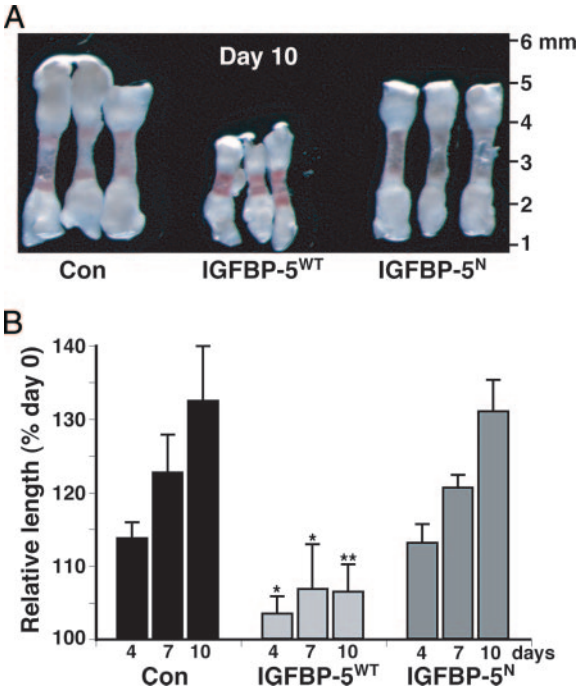

Fig. 6. IGFBP-5 Inhibits Metatarsal Bone Growth by IGF-Dependent Mechanisms

Neonatal mouse metatarsals were treated with purified IGFBP-5 (200 nM) and incubated in culture for 10 d as described in *Materials and Methods*. Bone length was measured at different time points. A, Representative images of metatarsal bones after incubation with control medium (Con) or with added IGFBP-5^{WT} or IGFBP-5^{N} for 10 d; B, change in metatarsal bone length at d 4, 7, and 10 compared with d 0 (mean ± SD, n = 6; *, $P < 0.01$ *vs.* Con; **, $P < 0.001$ *vs.* Con).

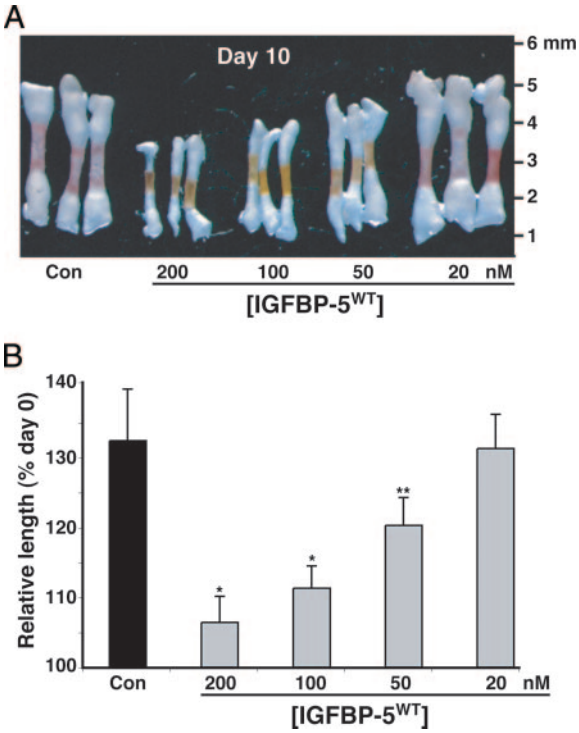

Fig. 7. Concentration-Dependent Inhibition of Metatarsal Bone Growth by IGFBP-5

A, Representative images of metatarsal bones at d 10 after incubation with control medium (Con) or with different concentrations of IGFBP-5^{WT} added at d 0; B, relative change in metatarsal bone length at d 10 compared with d 0 (mean ± SD, n = 5; *, $P < 0.001$ *vs.* Con; **, $P < 0.05$ *vs.* Con).

such as the vitamin D receptor (55) and four and a half LIM protein-2 (56), resulting in stimulation of osteoblast differentiation and function. To address this possibility, we used adenovirus-mediated gene transfer to deliver IGFBP-5^{WT} or IGFBP-5^{N} to BMP-2-treated mesenchymal precursors but observed the same spectrum of inhibitory outcomes as was seen with addition of purified IGFBP-5 to culture medium. Namely, IGFBP-5^{WT} blocked differentiation, and IGFBP-5^{N} was inert. We thus conclude that the mode of delivery does not influence the ability of IGFBP-5 to inhibit BMP-2-mediated osteogenic differentiation but cannot otherwise explain the divergent outcomes between our studies and those of other groups.

IGFBP-5 Inhibits Each Phase of Osteoblast Differentiation and Maturation

Bone cell differentiation may be divided into stages, beginning with commitment to the osteoblastic lineage, which is characterized initially by expression of Runx-2 (49, 57) and subsequently other transcription factors, like activating transcription factor-4 and Osx (3, 57), and continuing with production of other bone-specific proteins, including AP and Ocn, and in mature osteoblasts, mineralization of extracellular matrix (1, 50). We find that IGFBP-5 is able to block osteoblast differentiation at each step and can inhibit progression to the next stage (Fig. 5). Of particular note are results seen in Fig. 5C, showing that addition of IGFBP-5 on d 7 inhibited the normal up-regulation of Ocn gene expression, despite having no effect on mRNAs encoding bone-specific transcription factors. This raises the speculation that IGF actions in osteoblasts may facilitate transcription factor function and will be a topic for future investigation. Additionally, we demonstrate that IGFBP-5 impairs endochondral ossification in short-term metatarsal cultures (Fig. 8), results that are consistent with effects of targeted overexpression of IGFBP-5 or deletion of the IGF-I receptor in mature osteoblasts in transgenic mice (7, 37), which led to diminished mineralization but normal osteoid deposition. Mineralization involves accumulation of calcium phosphate in the extracellular matrix and is achieved by active transport of calcium and inorganic phosphate into osteoblasts, the subsequent release of matrix vesicles, and nucleation and eventual deposition of hydroxyapatite granules in the osteoid (58, 59). Multiple factors control mineralization, including the sodium-dependent phosphate transporter, Pit-1, which has been shown to be regulated by IGF-I in osteo-

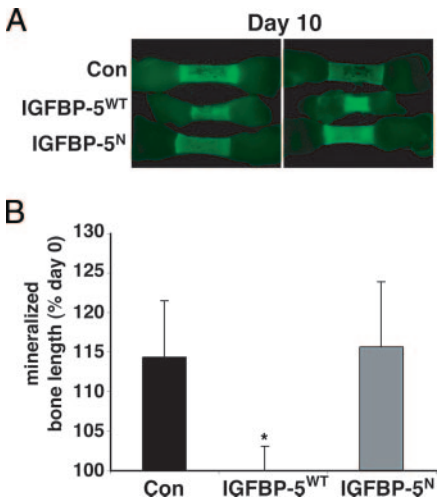

Fig. 8. IGFBP-5 Inhibits Metatarsal Bone Mineralization

Bones were labeled with calcein as described in *Materials and Methods*. A, Fluorescent images showing calcein-labeled mineralizing zone in metatarsal bones after a 10-d incubation in control culture medium (Con) or with added purified IGFBP-5^{WT} or IGFBP-5^{N}; B, change in length of calcein-labeled mineralizing zone at d 10 compared with d 0 (mean ± SD, n = 5; *, $P < 0.05$ *vs.* Con).

blasts *in vitro* (60, 61). Our model systems thus provide opportunities for discerning the biochemical and molecular mechanisms of IGF action during different phases of bone development and maturation. Because the process of endochondral ossification is also recapitulated during fracture healing (62, 63), understanding these mechanisms has the potential to lead to better therapeutic outcomes.

How Do IGF and BMP Signaling Pathways Collaborate during Osteoblast Differentiation?

Despite the profound inhibitory effects of IGFBP-5 on BMP-2-mediated osteoblast differentiation, the early events in BMP signaling appear to be intact. Phosphorylation of Smads 1, 5, and 8 is undisturbed by IGFBP-5, although induction of Smad-activated genes, such as Runx-2, is impaired (Figs. 2 and 3). IGF-I and IGF-II bind to and activate the IGF-I receptor, which through a series of adaptor proteins stimulates several intracellular signal transduction pathways (5). IGF-mediated signaling through phosphatidylinositol-3 (PI3)-kinase and Akt has been linked to differentiation of other cell types, including skeletal muscle (64–66). In this context, pharmacological inhibition of PI3-kinase or dominant-negative Akt has been shown to block BMP-2-induced osteogenesis (67–69), possibly by interfering with a positive feedback loop involving Runx-2 (70), and mice lacking Akt-1 and Akt-2 are osteopenic (71). In addition, because osteoblast-specific deletion of Pten, a negative regulator of PI3-kinase (72, 73), led to enhanced bone density in mice (74), the evidence supporting an important role for the PI3-kinase-Akt pathway in bone development and maturation is strong. However, because in other studies inhibition of the Mek-Erk pathway but not the PI3-kinase-Akt pathway has been found to impair BMP-2-mediated osteoblast differentiation (75, 76), the mechanisms of IGF-stimulated osteoblast differentiation and collaboration with BMP-2-activated pathways remain to be fully defined.

In summary, we have shown that IGFBP-5 is a potent inhibitor of osteoblast differentiation, bone growth, and mineralization through its ability to neutralize IGF actions by high-affinity growth factor binding and presumptive sequestration away from the IGF-I receptor. Our results point to a key role for IGF-mediated signaling in all phases of osteoblast development and function and raise a challenge to define the mechanisms of collaboration with BMPs.

MATERIALS AND METHODS

Reagents and Antibodies

DMEM, fetal calf serum, horse serum, and PBS were purchased from Mediatech-Cellgrow (Herndon, VA). Ascorbic acid, β-glycerol phosphate, sodium orthovanadate, Alizarin red, and heparin agarose were from Sigma-Aldrich (St. Louis, MO). Superscript III first-strand synthesis kit and trypsin/EDTA solution were obtained from Invitrogen (Carlsbad, CA). QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate tablets and protease inhibitor tablets were from Roche Applied Sciences (Indianapolis, IN), and okadaic acid was from Alexis Biochemicals (San Diego, CA). Dox (Clontech, Palo Alto, CA) was used at a final concentration of 1 mg/ml. The BCA protein assay kit and Gelcode blue staining solution were from Pierce Biotechnologies (Rockford, IL). TransIT-LT-1 was from Mirus Corp. (Madison, WI). Biotin-conjugated heparin was from Calbiochem, EMD Biosciences (San Diego, CA), Immobilon-FL was from Millipore Corp. (Billerica, MA), and AquaBlock EIA/WIB solution from East Coast Biologicals (North Berwick, ME). Restriction enzymes, buffers, ligases, and polymerases were from Roche Applied Sciences, BD Biosciences (Clontech) and Fermentas (Hanover, MD). Biotinylated human IGF-II and R^3IGF-I were from GroPep (Adelaide, Australia). Polyclonal antibodies were from the following: anti-Smad-1, anti-Akt, and anti-phospho-Akt (Ser^{473}) from Cell Signaling Technology (Beverly, MA); anti-IGFBP-5, anti-phospho-Smad, anti-phosphotyrosine, and anti-IGF-I receptor β-subunit from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-BMP-2 from R&D Systems (Minneapolis, MN). Goat antirabbit IgG-IR800 and goat antimouse IgG-IR680 were from Rockland Immunochemical Inc. (Gilbertsville, PA), and donkey anti-goat IgG-IR680 was from Molecular Probes (Eugene, OR). Other chemicals were reagent grade and were purchased from commercial suppliers.

Generation of Recombinant Adenoviruses

Recombinant adenoviruses encoding β-galactosidase (Ad-β-gal), the tetracycline transactivator protein (Ad-tTA), mouse IGFBP-5 (Ad-IGFBP-5^{WT}), and a modified mouse IGFBP-5 with low affinity for IGF-I (Ad-IGFBP-5^{N} mutant) have been described (44). The entire coding region of BMP-2 mRNA was cloned by RT-PCR from primary rat calvarial osteoblasts as two overlapping cDNA fragments that were joined together at

Table 1. Primers Used for RT-PCR of RNA

Gene	Location	DNA Sequence (5′–3′)	Size (bp)
Dlx-5	Exon 2	CTGGCCGCTTTACAGAGAAG	220
	Exon 3	CTGGTGACTGTGGCGAGTTA	
Ocn	Exon 1	CAAGTCCCACACAGCAGCTT	200
	Exon 2	AAAGCCGAGCTGCCAGAGTT	
Osx	Exon 1	ACTCATCCCTATGGCTCGTG	238
	Exon 1	GGTAGGGAGCTGGGTTAAGG	
Runx-2	Exon 4	GCCGGGAATGATGAGAACTA	200
	Exon 5	GGACCGTCCACTGTCACTTT	
S17	Exon 2	ATCCCCAGCAAGAAGCTTCGGAACA	332
	Exon 5	TATGGCATAACAGATTAAACAGCTC	

a unique internal *Hin*dIII site and subcloned into pBluescript$^+$ (pBSSK$^+$; Stratagene) at 5′ *Sal*I and 3′ *Eco*RV sites (*underlined*). The following primers were used: 5′-half sense strand, 5′-CTAAAGGTCGACCATGGTGGCCGG-3′, and antisense, 5′-GTGTTCTGAGTCACTAACCTGGTG-3′; and 3′-half sense, 5′-GGGAAAACTTCCCGACGCTTCTTC-3′, and antisense, 5′-CGTAGCGATATCCTAGCGACAC-CCGCAACCCTC-3′. After DNA sequence verification and subcloning into the pShuttle CMV vector, Ad-BMP-2 was prepared, purified, and titered as described (64).

Purification of IGFBP-5

C3H10T1/2 mouse embryonic fibroblasts (catalog no. CCL226; American Type Culture Collection, Rockville, MD) were incubated at 37 C in humidified air with 5% CO_2 in DMEM with 10% fetal calf serum. Cells were infected at about 50% of confluent density with Ad-tTA plus either Ad-IGFBP-5^{WT} or Ad-IGFBP-5^N (multiplicities of infection of 500). The next day, medium was replaced with DMEM plus 0.5% horse serum, and after conditioning for 48 h, secreted IGFBP-5 was purified by heparin-affinity chromatography (44) and stored in aliquots at −80 C until use.

Osteogenic Differentiation

C3H10T1/2 cells were incubated as above. Cells were infected at about 50% of confluent density with Ad-β-gal or Ad-BMP-2 with or without Ad-tTA (multiplicities of infection of 500) and 24 h later incubated in osteogenic media (OM: DMEM plus 10% fetal calf serum plus 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate) (31, 37) with or without addition of Ad-IGFBP-5^{WT} or Ad-IGFBP-5^N (multiplicities of infection of 500) with or without Dox. OM with or without Dox was replaced every 48 h for up to 15 d. Alternatively, purified IGFBP-5^{WT} or IGFBP-5^N was added to Ad-BMP-2-infected cells in OM. OM with or without IGFBP-5 was replaced every 48 h for up to 12 d.

Mouse Metatarsal Bone Culture

Pregnant mice were housed at the Oregon Health and Science University (OHSU) Animal Care Facility on a 12-h light, 12-h dark schedule with free access to food and water and received care according to National Institutes of Health guidelines. The OHSU Committee on Animal Care and Use approved all experiments involving mice. Metatarsal bones were surgically isolated from newborn C57BL/6 mice (d 0–3 after birth) and were incubated in DMEM containing 0.5% BSA, 50 μg/ml ascorbic acid, 1 mM β-glycerol phosphate, and 100 μg/ml penicillin-streptomycin solution at 37 C in humidified air with 5% CO_2 (51, 54). Purified IGFBP-5^{WT} or IGFBP-5^N was added to medium 1 d later, and metatarsals were cultured for up to 10 d. Imaging was performed at d 1, 4, 7, and 10 using a Lieca MZ FLIII microscope attached to a Nikon DXL1200 camera. For staining of calcium deposition, metatarsals were incubated in media containing calcein (500 ng/ml) for 2 h, followed by three rinses with PBS. Fluorescent images were captured with a Nikon Eclipse T300 fluorescent microscope by an attached Roper Scientific Cool Snap FX CCD camera using an Apple Macintosh PowerPC computer running IP Labs Scientific Image Processing software v3.9.4r2 (Scanalytics, Inc., Fairfax, VA).

Protein Extraction, Immunoblotting, and Ligand Blotting

Cell lysates and conditioned cultured medium were prepared as described (64), and aliquots were stored at −80 C until use. Protein samples (30 μg/lane) were resolved using reducing SDS-PAGE and transferred to Immobilon-FL. After blocking with AquaBlock for 1 h at 20 C, membranes were incubated sequentially with primary and secondary antibodies. Primary antibodies were used at the following dilutions: anti-IGFBP-5 (1:1000), anti-Akt (1:1000), anti-phospho-Akt (Ser^{473}) (1:1000), anti-BMP-2 (1:1000), anti-phospho-Smad (1:1000), anti-Smad (1:1000), and the appropriate conjugated secondary antibody at 1:5000. For immunoprecipitation studies, cellular protein lysates (200 μg) were incubated overnight at 4 C with phosphotyrosine antibody (1 μg), followed by SDS-PAGE and immunoblotting with an IGF-I receptor β-subunit antibody (1:1000 dilution), and other steps as described (44). For ligand blotting, SDS-PAGE under nonreducing conditions was used to resolve conditioned medium. After transfer to Immobilon-FL and blocking in Aquablock, membranes were incubated with biotin-conjugated IGF-II (100 ng/ml) in 50% AquaBlock, 50% PBS, and 0.1% Tween 20 for 16 h at 4 C. After binding, membranes were washed with Tris-buffered saline with Tween 20, followed by incubation with IR800-conjugated streptavidin (1:5000 dilution). Images were capture with the LiCoR Odyssey Infrared Imaging System, and analyzed with version 1.2 software (LiCoR, Lincoln, NE).

Transient Transfections and Luciferase Reporter Gene Assays

A promoter-reporter plasmid containing six copies of OSE-2 from the osteocalcin gene promoter (49, 77) was prepared by annealing the following primers and cloning the double-stranded DNA into TK Luc at 5′ *Hin*dIII and 3′ SalI sites (overhangs *underlined*): top strand, 5′-*AGCTA*ACCACAA-ACCACAAACCACAAACCACAAACCACA-3′, and bottom strand, 5′-*TCGA*TGTGGTTTGTGGTTTGTGGTTTGT-GGTTTGTGGTTTGTGGTT-3′. C3H10T1/2 cells were seeded in 12-well tissue culture dishes (1×10^5 cells per well) and transfected 24 h later with 100 ng promoter-reporter DNA using TransIT-LT-1. One day later, cells were infected se-

quentially with recombinant adenoviruses as above. After incubation in OM for 48 h with or without Dox, cells were harvested and lysates used for luciferase assays (78). Results were normalized for cellular protein concentrations.

AP Staining and Activity

Cells were washed with PBS, fixed with 70% ethanol for 10 min, and incubated with 500 ml nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate solution (one tablet in 10 ml distilled water) for 20 min at 20 C (79). After three washes with distilled water, images were captured using the Odyssey Infrared Imaging System. AP activity was determined spectrophotometrically at 405 nM after incubating cell lysates (10 μg) with 1 mg/ml solution of *p*-nitrophenyl phosphate (50 μl) in a 96-well format for 20 min at 20 C (80).

Alizarin Red Staining

Cells were fixed in 70% ethanol for 10 min, and mineralized nodules were stained with 0.1% Alizarin red solution (pH 4.1–4.5) for 1 min at 20 C (80). Images were obtained by scanning plates on a Canon flatbed scanner or with the Odyssey Infrared Imaging System. Adobe Photoshop was used for image processing and editing. Mineralization was quantified after capturing images at $\times$200 magnification with a Nikon Eclipse T300 fluorescent microscope and a Roper Scientific Cool Snap FX CCD camera, as outlined above. Data analysis was performed with NIH Image 1.63.

Analysis of Gene Expression

Total RNA was isolated, concentrations determined at 260 nm, and quality assessed by agarose gel electrophoresis (64). RNA (2 μg) was reverse transcribed with Superscript III first-strand synthesis kit using oligo (dT) primers in a final volume of 20 μl. PCR was performed with 1 μl cDNA and primer pairs listed in Table 1 after establishing the linear range of product amplification in pilot studies. Cycle numbers ranged from 20–30. Results were analyzed using Quantity One Software (Bio-Rad) after agarose gel electrophoresis.

Statistical Analysis

Data are presented as mean $\pm$ SD. Statistical significance was calculated using a paired Student's *t* test. Results with *P* values < 0.05 were considered significant.

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