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Identification and characterization of novel IGFBP5 interacting protein: evidence IGFBP5-IP is a potential regulator of osteoblast cell proliferation

Yousef G. Amaar¹, Blanca Tapia¹, Shin-Tai Chen^{1,2}, David J. Baylink^{1,2,3}, and Subburaman Mohan^{1,2,3,4}

¹Musculoskeletal Disease Center, Jerry L. Pettis Veterans Administration Medical Center, Loma Linda

²Department of Medicine, Loma Linda University, Loma Linda, California

³Department of Biochemistry, Loma Linda University, Loma Linda, California

⁴Department of Physiology, Loma Linda University, Loma Linda, California

Abstract

Insulin-like growth factor binding protein-5 (IGFBP5) is a multifunctional protein, which acts not only as a traditional binding protein, but also functions as a growth factor independent of IGFs to stimulate bone formation. It has been predicted that the intrinsic growth factor action of IGFBP5 involves binding of IGFBP5 to a putative receptor to induce downstream signaling pathways and/or nuclear translocation of IGFBP5 to influence transcription of genes involved in osteoblast cell proliferation/differentiation. Our study identified proteins that bound to IGFBP5 using IGFBP5 as bait in a yeast two-hybrid screen of the U2 human osteosarcoma cell cDNA library. One of the clones that interacted strongly with the bait under high-stringency conditions corresponded to a novel IGFBP5 interacting protein (IGFBP5-IP) encoded by a gene that resides in mouse chromosome 10. The interaction between IGFBP5-IP and IGFBP5 is confirmed by in vitro coimmunoprecipitation studies that used pFlag and IGFBP5 polyclonal antibody, and cell lysates overexpressing both IGFBP5-IP and IGFBP5. Northern blot and RT-PCR analysis showed that the IGFBP5-IP is expressed in both untransformed normal human osteoblasts and in osteosarcoma cell lines, which are known to produce IGFBP5. To determine the roles of IGFBP5-IP, we evaluated the effect of blocking the expression of IGFBP5-IP on osteoblast proliferation. We found that using a IGFBP5-IP-specific small interfering-hairpin plasmid resulted in a decrease in both basal and IGFBP5-induced osteoblast cell proliferation. On the basis of these findings, we predict that IGFBP5-IP may act as intracellular mediator of growth promoting actions of IGFBP5 and perhaps other osteoregulatory agents in bone cells.

Keywords

alternate splicing; yeast two-hybrid screen; small interfering RNA

Insulin-Like Growth Factors (IGFs) play a vital role in regulating cell proliferation, differentiation, and apoptosis (20,21,26). IGFs regulatory effects in bone cells are controlled

by their cognate receptors, IGF binding proteins (IGFBPs), and IGFBP proteases. It has been shown that IGFBPs both regulate the activity of IGFs and act independently of ligands (7,23,24,31). Although much is known about the mechanisms by which IGFBPs modulate IGF actions, little is known about the molecular mechanism for IGF-independent action of IGFBPs.

IGFBP5 stimulates both osteoblast cell proliferation and activity in vitro (4,4a,18,25) and recent findings demonstrate that IGFBP5 itself is a growth factor with cellular effects that are IGF independent (22). In support of this, it has been shown that IGFBP5 treatment increased bone formation parameters in vitro and in vivo in osteoblasts derived from IGF-I knockout mice (18). The IGF-independent effects of IGFBP5 have also been confirmed using transgenic mouse models overexpressing IGFBP5 (26a). Evidence suggests that IGFBP5 binds to a putative receptor on the osteoblast cell surface and stimulates downstream signaling pathways (3,21,28). In addition, IGFBP5 contains a nuclear localization sequence that mediates the transport of IGFBP5 to the cell nucleus (28,29) where it may affect gene transcription. IGFBP5 has also demonstrated IGF-receptor-independent effects in normal human intestinal smooth muscle cells via the activation of the P38 MAP kinase and Erk1/2, leading to a stimulation of cell proliferation and secretion of IGF-I (13).

To understand the molecular mechanism through which IGFBP5 regulates bone formation via an IGF-independent pathway, it is essential to identify the cellular proteins that interact with IGFBP5. These proteins could be IGFBP5 receptors, nuclear proteins, as well as signaling proteins that mediate IGFBP5-dependent actions. We therefore utilized the yeast two-hybrid assay (8) screen to identify those proteins that bind to IGFBP5 using human IGFBP5 as bait for screening a human osteosarcoma U2 cDNA library. We (2) recently reported on IGFBP5 interaction with the four and a half LIM domain protein 2 (FHL2) and demonstrated that FHL2 binds IGFBP5, but not IGFBP-4 or IGFBP-6. In this article, we report on the identification of a novel IGFBP5 interacting protein (IGFBP5-IP) with the use of the yeast two-hybrid assay. We show that IGFBP5-IP is expressed in various bone cell lines and it shared some sequence identity with a RIKEN cDNA clone (Genebank accession no. NM_172788.1). To define the function of this novel IGFBP5-IP in vitro, we used the small interfering RNA (siRNA) technique (6,10,15,32) to study the consequence of blockage of IGFBP5-IP expression on cell proliferation. We found that reduction in IGFBP5-IP expression reduced cell growth in multiple osteoblast cell lines, demonstrating that IGFBP5-IP could function to promote cell proliferation.

MATERIALS AND METHODS

Yeast two-hybrid screen

IGFBP5 was used as bait to screen a human osteosarcoma cell cDNA library as previously described (2).

Osteoblast cell culture

Normal human osteoblasts were isolated as described (2,16) from calvaria and rib bone specimens obtained from the Cooperative Human Tissue Network, which is supported by the National Cancer Institute. Cell culture was carried out as previously described (2,16).

RNA isolation and Northern blot analysis

Total RNA from human osteosarcoma U2 cell lines was isolated using the TriZOL reagent (Invitrogen, Carlsbad, CA) or using the RNAeasy kit (Qiagen, Valencia, CA). Northern blot analysis was carried out as previously described (2).

Expression analysis of IGFBP5-IP gene in bone cells

To determine the expression of the *clone A* gene in different bone cell lines, the following *clone A*-specific primers were used: forward, 5'-GCGCACTCAGACTTCTCCAT-3', and reverse, 5'-TGCTAGAGCTCTGCTAC-3'. One microgram of total RNA was used for reverse transcription reaction using the Omniscript kit (Qiagen). One micro-liter of the RT reaction was used for PCR using the HotStart master mix (Qiagen). The PCR reactions were run at following conditions: 95°C for 15 min, 95°C for 1 min, 60°C for 30 s, and 72°C for 30 s for 35 cycles.

Construction of IGFBP5-IP silencer plasmid

On the basis of the coding sequence alignment of IGFBP5-IP with the RIKEN cDNA clone sequence (Genebank accession no. NM_172788.1), we designed siRNA duplexes specific to IGFBP5-IP and the RIKEN cDNA clone with the use of Qiagen software (available at www.Qiagen.com). We designed a siRNA-1323 duplex with a perfect match to both IGFBP5-IP and the RIKEN cDNA clone and designed a siRNA-207 duplex that is specific to RIKEN cDNA clone. The sequence of the siRNA-1323 insert is the following: 5'-TCGAGCCAGATGACAAGAAAACgagtactgGTTTTTCTTGTCATCTGGCTTTTT-3', 3'-CGGTCTACTGTTCTTTTTGctcatgacCAAAAAGAACAGTAGACCGAAAAAGATC-5'. The sequence for siRNA-207 insert is the following: 5'-TCGACTATGATGCCCTTTGTCCgagtactgGGACAAGAGGGGCATCATAGTTTTT-3' and 3'-GATACTACGGGAGAACAGGctcatgac-CCTGTTCTCCCGTAGTATCAAAAAGATC-5'.

The sequence of random siRNA insert is 5'-TCGAGTACAACCTTCGATTTCGACGttcaagagACGTCGAARCGAAGTTGTACAAAAA-3' and 3'-CTAGTTTTTGTACAACCTTCGATTTCGACGtctctttaaCGTGAATCGAAGTTGTAC-5'. The primers were annealed and cloned into the pSuppressorNeo vector, as recommended by the supplier (Imgenex, San Diego, CA). The purified vectors containing inserts were identified by digestion with *ScaI* and DNA sequencing.

Treatment of cells with siRNA molecules

Cells were seeded at 5,000 per well in 96-well plates or 35,000 cells per well in 6-well plates in DMEM, supplemented with 10% calf serum 24 h before treatment with siRNA vectors (siRNA-207 and siRNA-1323) and vector alone. MC3T3-E1 (mouse osteoblast), MG63, HSaOs, and LSaOS cells (human osteosarcoma) were transfected with 1 µg of siRNA vector or vector alone with the use of Effectine transfection reagent, as recommended by the supplier (Qiagen). The transfection medium was replaced with a fresh DMEM containing 10% calf serum medium and cells were incubated for additional 48 h before performing the AlamarBlue Assay and RNA extractions.

AlamarBlue assay

The biological effect on cells treated with siRNA molecules was measured by the AlamarBlue assay. AlamarBlue is reduced by reactions innate to cellular metabolism and therefore provides an indirect measure of viable cell number (AccuMed International, Westlake, OH). Seventy-two hours after transfection, cells treated in 96-well plates were rinsed with PBS, and the medium was replaced with 100 µl of 1× AlamarBlue diluted in phenol red-free DMEM. Direct light was avoided while the dye was used, and the plates were incubated for 4 h at 37°C.

Fluorescence was determined with the use of a fluorescent plate reader (Fluorolite 1000; Dynex Technologies, Chantilly, VA).

³H-Thymidine incorporation

MC3T3-E1 mouse osteoblasts and LSaOS human osteosarcoma cells were plated in α MEM containing 1% calf serum at a density of 2,000 cells per well in 96-well culture plate. Twenty-four hours later, cells were treated with siRNA 1323, siRNA 207, or random siRNA control, as described above. Fresh serum-free DMEM containing 0.1% BSA was added 24 h later and cultures were treated with or without 100 ng/ml IGFBP5. Eighteen hours later, 0.25 μ ci ³H-thymidine was added and incubated for 6 h before termination. The amount of ³H-thymidine incorporated into DNA was evaluated as previously described (22).

RNA isolation and RT-PCR analysis

MC3T3-E1 cells were transfected with 1 μ g/ml of siRNA-207 vector, siRNA1323 vector, and vector alone using Effectine (Qiagen). Seventy-two hours posttransfection, cells were collected and total RNA was extracted using a RNA Microprep kit (Stratagene, La Jolla, CA). Five hundred nanograms of total RNA were used to prepare cDNA with Omniscript kit (Qiagen). Five microliters of the RT reaction were used for PCR using the HotStart master mix (Qiagen). The PCR reactions were run at the following conditions: 95°C for 15 min, 95°C for 1 min, 60°C for 30 s, and 72°C for 30 s for 35 cycles. We used three sets of primers: the primer set CAF2/CAR285 (gives 390-bp PCR product: this sets of primers used to detect IGFBP5-IP expression in human bone cells), the primers set CAF2/CAR1 (gives a product of 610 bp: this set of primers used to detect IGFBP5-IP expression in MC3T3 cells), and the primer set RIKEN-pf596F/pR618 (gives 350-bp product: this set of primers is used to detect the RIKEN expression in MC3T3 cells). The sequences of the primer sets used were the following: pCAF2: 5' GCGCACTCAGACTTCTCCAT3'; pCAR285: 5'AGAGGTGGCTCGGCAGTGAA 3'; pCAR1: 5' GGCATTGAACAGAGGCAGAA 3'; RIKEN-pF596F: 5' TCGATCAACGTGTACCTGGCTCTC3'; and RIKEN-pR618: 5' GGTGCTGCATGTGATCCT 3'.

Overexpression of pFlag-IGFBP5-IP and murine leukemia virus-IGFBP5 constructs in U2 cells and coimmunoprecipitation

U2 cells were first transfected with murine leukemia virus-IGFBP5 construct, as previously described (2), and cells were checked for overexpression of IGFBP5 protein using Western blot analysis. U2 cells overexpressing IGFBP5 were subsequently transfected with pFlag-IGFBP5-IP construct using 10 cm tissue culture plates. Forty-eight hours after transfection, the cells were collected and cell lysates were prepared as previously described (2). Coimmunoprecipitation was carried out using Pflag and IGFBP5 antibodies, as previously described (2).

RESULTS

Identification of a novel IGFBP5 interacting protein

IGFBP5 was used as bait to screen a human osteosarcoma cDNA library using the yeast two-hybrid system, as previously described (2). We obtained ~50 positive clones during the first screening, out of which approximately one-half of the clones were found to be positive in subsequent reconfirmation experiments. For subsequent characterization of positive clones, we focused on those clones, which grew vigorously in the reconfirmation test. In this study, we identified a 0.8-kb cDNA clone (*clone A*) (Table 1) that exhibited a strong interaction with IGFBP5 under high-stringency conditions. The *clone A* cDNA sequence did not completely match with any known sequence, but it showed some sequence identity with a mouse RIKEN

cDNA clone sequence (GeneBank Accession no. NM_172788.1). Because *clone A* cDNA did not match any known gene sequence, we have named it IGFBP5-interacting protein (IGFBP5-IP). The IGFBP5-IP cDNA encodes an open reading frame of 257 amino acids, and it contains a stop codon and a poly A tail at the 3'-end (Fig. 1). The mouse RIKEN cDNA clone showed an 82% sequence homology (1,086/1,316 bp) with a human mRNA for FLJ00204 protein.

Sequence comparison of IGFBP5-IP vs. RIKEN cDNA clone

Because the IGFBP5-IP cDNA sequence showed partial identity to RIKEN cDNA clone, we next determined whether our IGFBP5-IP cDNA and the RIKEN cDNA clone sequence are derived from the same gene. We used our IGFBP5-IP cDNA (Fig. 1) and the RIKEN cDNA sequences to search the mouse genome using the UCSC genome bioinformatics web site (<http://genome.ucsc.edu>). Interestingly, the analysis of the sequence alignments has shown that the first segment (exons 1 and 2, designated as exon 6A and exon 7, respectively) of the IGFBP5-IP sequence shares a 429 nucleotide identity with the RIKEN cDNA clone sequence that includes part of exon 6 and the entire exon 7 sequence; and the second segment (exon 3 designated as exon 8A) of the IGFBP5-IP sequence falls within intron 7 of the RIKEN cDNA genomic sequence on mouse chromosome 10 (Fig. 2, A and B). The sequence analysis data suggest that IGFBP5-IP and RIKEN cDNA may represent alternatively spliced forms of the same gene. It should be noted that primer extension analysis using IGFBP5-IP and RIKEN cDNA forward and reverse primers revealed no additional regions of identity upstream of exon 1 of the IGFBP5-IP sequence (data not shown).

IGFBP5-IP gene is expressed in bone cells

To confirm that the IGFBP5-IP transcript is expressed in bone cells, we have performed RT-PCR experiments using total RNA from untransformed normal human bone calvaria (HBC) cells and human osteosarcoma cell lines (MG63 and U2). IGFBP5-IP cDNA specific primers amplified a 390-bp PCR product in all three cell types (Fig. 3A). The sequence of the RT-PCR product from HBC, MG63, and U2 cells matched our IGFBP5-IP cDNA sequence (data not shown), suggesting that these cells express the IGFBP5-IP gene. These findings provide experimental support to our hypothesis that IGFBP5-IP is a novel gene and it is expressed in human bone cells.

In addition, we have also carried out Northern blot analysis using total RNA isolated from U2 cells and used IGFBP5-IP cDNA as a probe. The probe detected a 1.5 kb band (Fig. 3B) in the U2 cell line, thus providing further evidence that the IGFBP5-IP gene is expressed in bone cells. It is unknown whether the larger size of the IGFBP5-IP transcript found in the Northern blot analysis (1.5 kb), compared with the cDNA clone (0.8 kb), is due to the long untranslated region in the IGFBP5-IP transcript.

IGFBP5/IGFBP5-IP interaction determined by coimmunoprecipitation

To further confirm interaction between IGFBP5-IP and IGFBP5 observed in the yeast two-hybrid assay, cell lysates from U2 cells overexpressing IGFBP5 and pFlag-IGFBP5-IP were immunoprecipitated with pFlag antibody and then probed with IGFBP5 antiserum with the use of Western immunoblot analysis (Fig. 4). IGFBP5 was immunoprecipitated by pFlag antibody in the presence of IGFBP5 and pFlag-IGFBP5-IP. The weak IGFBP5 band that was seen in the presence of normal mouse IgG is likely due to nonspecific interactions between IGFBP5 and normal mouse IgG or between IGFBP5 and protein A sepharose beads. The immunoprecipitation data together with the yeast two-hybrid data provide evidence that IGFBP5 and IGFBP5-IP do interact. However, the physiological significance of their interaction in bone cells remains to be determined.

Silencing of IGFBP5-IP expression reduces osteoblast cell number

Because IGFBP5 regulates proliferation and differentiation of bone cells, we hypothesize IGFBP5-IP to exhibit similar biological effects if it were to be involved in mediating IGFBP5 signaling in osteoblasts. We therefore constructed a siRNA vector (pSuppressorNeo-siRNA-1323) specific for IGFBP5-IP and RIKEN cDNA mRNAs and tested it in multiple osteoblast cell lines. We first tested the effect of siRNA-1323 duplex on expression of IGFBP5-IP and RIKEN mRNA. Figure 5 shows that siRNA-1323 reduced expression of IGFBP5-IP, as well as RIKEN transcript in MC3T3-E1 mouse osteoblasts. This is not surprising because siRNA-1323 is 100% identical to both IGFBP5-IP and RIKEN cDNA clone. In contrast, siRNA-207 that is specific to RIKEN cDNA clone decreased expression of RIKEN transcript, but not IGFBP5-IP transcript. Treatment with neither siRNA-1323 nor siRNA-207 altered expression of the actin gene (Fig. 5).

We next tested the effects of siRNA-1323 duplex on the proliferation of MC3T3-E1 mouse osteoblasts, MG63, LSAOs, and HSAOS human osteoblast cell lines. Our data show that treatment of cells with siRNA-1323 vector significantly reduced cell proliferation, as determined using the AlamarBlue assay, compared with cells treated with the vector alone (Fig. 6, A–D). We found that the treatment of MC3T3-E1 cells with siRNA-207 had no effect on cell number compared with cells treated with the vector alone, as determined using the AlamarBlue assay (Fig. 6E).

siRNA-1323 blocks IGFBP5-induced cell proliferation

Treatment of MC3T3-E1 or LSAOs-2 cells with 100 ng/ml IGFBP5 significantly increased ³H-thymidine incorporation. IGFBP5-induced increase in ³H-thymidine incorporation was blocked by siRNA-1323 (Fig. 7). This effect was specific since neither random siRNA control nor siRNA-207 had any significant effect on IGFBP5-induced increase in ³H-thymidine incorporation in either cell type.

DISCUSSION

We used IGFBP5 fused to GAL binding domain as bait to screen a U2 human osteosarcoma cDNA library fused to the GAL activation domain (AD) in the expression vector pACT2 to identify candidate proteins that interact with IGFBP5 using the yeast two-hybrid system. One of the positive clones (*clone A*) is found to represent a novel gene that has not yet been characterized. The clone contained a full-length cDNA sequence encoding 257 amino acids and strongly interacted with IGFBP5 under high-stringency conditions, as demonstrated by the two-hybrid assay and further confirmed by immunoprecipitation studies. The novel protein encoded by *clone A* cDNA shows a 100% sequence identity with the mouse genomic sequence residing on chromosome 10; however, it did not show 100% sequence identity with any of the human sequence in the NCBI database. We have confirmed the expression of the IGFBP5-IP gene by Northern and RT-PCR analysis using human bone cells derived from calvaria (HBC), MG63, and U2 osteosarcoma cell lines as well as the mouse MC3T3-E1 cell line. On the basis of the finding that IGFBP5-IP is expressed in human osteoblasts in addition to mouse osteoblasts, we think that when all of the human genome sequence is accounted for, the chromosomal location for the IGFBP5-IP gene will be identified.

The interaction between IGFBP5 and IGFBP5-IP observed in yeast was confirmed by immunoprecipitation studies using U2 cell lysate overexpressing IGFBP5 and pFlag-IGFBP5-IP proteins, and antibodies directed against the pFlag-IGFBP5-IP and IGFBP5. Although the yeast two-hybrid assay and the in vitro coimmunoprecipitation experiments provide evidence that the interaction between IGFBP5 and IGFBP5-IP occurs, further studies are needed to prove that these two proteins bind under normal physiological conditions.

IGFBP5 has been shown to be a multifunctional protein that acts through IGF-dependent and IGF-independent mechanisms. In fact, other IGFBPs such as IGFBP-1 and -3 have also been shown to mediate their effects on a variety of cell types in part through IGF-independent mechanism (7,11,16,23,24,31). Several IGFBP-3 interacting proteins have also been discovered using the yeast two-hybrid assay (14,17,30), and we (2) have previously reported on the identification of FHL2 as a binding partner of IGFBP5. On the basis of our discovery that IGFBP5-IP interacts with IGFBP5, we hypothesized that IGFBP5-IP may mediate, in part, the stimulatory effect of IGFBP5 on osteoblast cell proliferation. Accordingly, we predicted that silencing of IGFBP5-IP expression in IGFBP5 producing cells should decrease proliferation. To test this prediction, we evaluated the consequence of silencing the expression of IGFBP5-IP by using siRNA on proliferation of osteoblast cell lines. Our findings in this study demonstrate that plasmid encoding a siRNA duplex (Si-1323) that is sequence specific to IGFBP5-IP caused a significant ($P < 0.05$) decrease in both basal and IGFBP5-induced cell proliferation. The relative contribution of IGFBP5-IP in mediating the biological effects of IGFBP5 in other cell types still needs to be determined.

In previous studies, we (1,2) have found that IGFBP5 interacted with FHL2, a potential transcription modulator and RASSF1C, a potential Ras effector. The significance of why IGFBP5 interacts with multiple signaling molecules can only be speculated on at this time. It has been shown that intermittent administration of IGFBP5 increased bone formation while it sustained transgenic overexpression of IGFBP5 decreased bone formation (25,26a), suggesting that IGFBP5's effects on bone formation are complex. IGFBP5 also has multitude of effects on osteoblasts influencing cell proliferation, differentiation, and apoptosis (4,18,22,25). Although we have not characterized the nature of IGFBP5 interactions with its newly identified intracellular partners at the physiological level, it is tempting to speculate that IGFBP5 interactions with each of these proteins may involve a unique pathway and thus could contribute to a distinct biological effect.

In conclusion, 1) IGFBP5-IP is a novel gene and it is located on mouse chromosome 10; 2) IGFBP5-IP is expressed in human and mouse osteoblasts; and 3) silencing of IGFBP5-IP gene expression decreases cell proliferation. Our finding that inhibition of IGFBP5-IP expression decreased IGFBP5-induced osteoblast cell proliferation is consistent with the possibility that IGFBP5-IP could act as an intracellular mediator of IGFBP5's mitogenic effects. The potential pathway(s) through which IGFBP5-IP might mediate IGFBP5's mitogenic effects will be investigated in future studies in our laboratory.

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 M Q H P A T S L P T G S C L
 GGC ACTCAGCCCAACCAACAGCCAGCCAGGCCGGGGATAACCACCATCCCAAC
 R H S A Q P T A S Q A G D T T I P
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 CCCCTGCGCACTCAGACTTCTCCATCCCGCCTGCCAGCACCGGCCTGAGACC
 P L R T Q T S P S R L P S T G L R
 CCGCTCTGTGGCGTCCCCACAGCATGGCCAGCAGTCCCCGGCACAGATGTGT
 P R S V A S P Q H G Q Q S P A Q M C
 CCCCCGCCAGCCATCCCCTTCACATCAGCGGCCTCCGCCATCACACCCCCCAA
 P R P A I P F T S A A S A I T P P
 TGTCAGTGCAGCCAATCTGAGCGGGGAGGTCCGAGGGACACCCATCAGCGG
 N V S A A N L S G E V G G T P I S
 CCTGTCCACACCCAGCCTCATCAACACGGGCTTCAAGCCAGATGACAAGAAA
 G L S T P S L I N T G F K P D D K K
 AACGAAAAGAGTTCCGGAGACAGCGTCAGAAGCCTTGGAACTGGAGGTGCA
 N E K S S G D S V R S L G T G G A
 GATGGTTGTGAGCCACCCCGTGGGTGCTGGGAATCAAACCCAGGTCCTCTGC
 D G C E P P R G C W E S N P G P L
 AAGAACAAATGCTCTTCACTGCCGAGCCACCTCTCCAGACCCCAAGATGCAC
 Q E Q M L F T A E P P L Q T P R C
 TTCTTTAGAGAGCAGTGACTTAAAAAGGAATACTGTGTATTGCTCCAGAATCC
 T S L E S S D L K R N T V Y C S R I
 TAAAGGCCAGATATGCCCGAGGCTGCAAACATCACAGCATCTGCACTGACCG
 L K A R Y A R G C K H H S I C T D
 TGTGTATGTAGCAGAGCATCTAGCA
 R V Y V C V C V C V C V C V C S R A S S
 CTGCAGGATGGACCAGTGATTCTGCCTCTGTTCAATGCCTTGCTCAGAAG**TAG**
 T A G W T S D S A S V Q C L A Q K *
 TAGGGCCGAGGCCAGTTGATAAATGGATTATTATACT**AAAAAAAAAAAAAAAA**

AAAA

Fig. 1. Insulin-like growth factor binding protein-5-interacting protein (IGFBP5-IP) cDNA sequence and its putative translated protein sequence are shown. The IGFBP5-IP cDNA encodes a putative protein of 257 amino acids. The initiation codon (ATG), the stop codon (TAG), and the polyA tail are shown in bold.

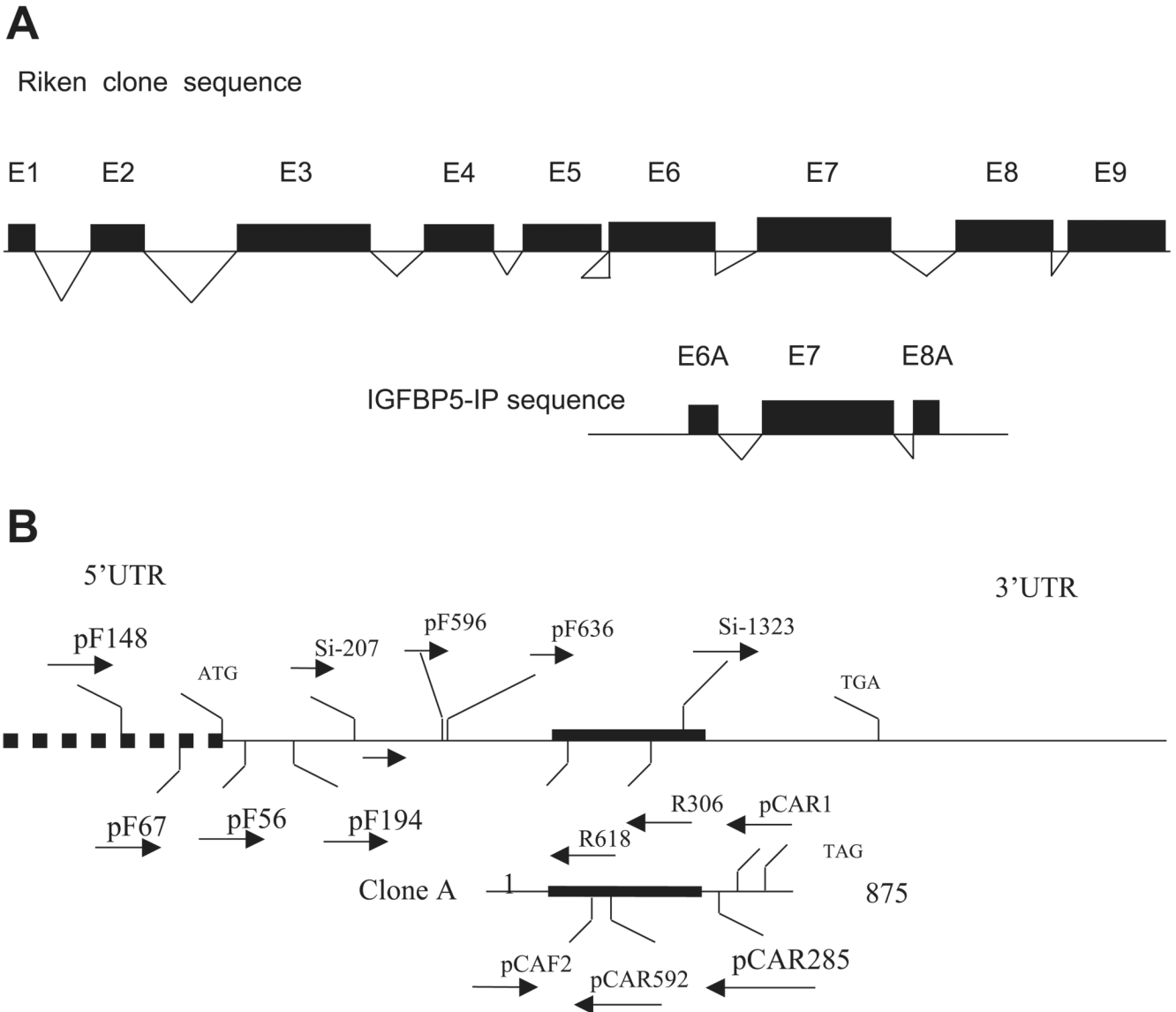


Fig. 2.

A: schematic diagram showing the region of homology for IGFBP5-IP and full-length coding/genomic sequence of the RIKEN-cDNA clone on mouse chromosome 10. The IGFBP5-IP cDNA sequence shares sequence identity with the last part of exon 6 and the entire sequence of exon 7 of the RIKEN cDNA (solid bars). The position of exon E8A of the IGFBP5-IP falls within intron 7 of the RIKEN gene sequence, suggesting that RIKEN cDNA clone and IGFBP5-IP are two alternatively spliced transcripts produced by the same gene. Open triangles show the positions of the introns within the RIKEN gene sequence. *B:* the position and direction of various forward and reverse primers specific to RIKEN and IGFBP5-IP cDNA sequences used for RT-PCR. →Forward primer←; reverse primer←-. pF, RIKEN-specific forward primer, pCAR, *clone A* (IGFBP5-IP) reverse primer, and pCAF, *clone A* (IGFBP5-IP) forward primer. The positions of small-interfering RNA (siRNA) that is specific to RIKEN cDNA (Si-207) and the siRNA that is common to both RIKEN cDNA and *clone A* (Si-1323) are also shown. The numbers indicate base pair positions that correspond to ATG start site of RIKEN cDNA. All RIKEN forward primers vs. RIKEN reverse primers produced PCR products of the expected size (results are not shown), whereas RIKEN-specific forward primers vs. IGFBP5-IP-specific

reverse primers (pCAR2 and pCAR285) did not produce any RT-PCR products, suggesting that IGFBP5-IP does not share regions of homology upstream of the IGFBP5-IP exon 6A sequence.

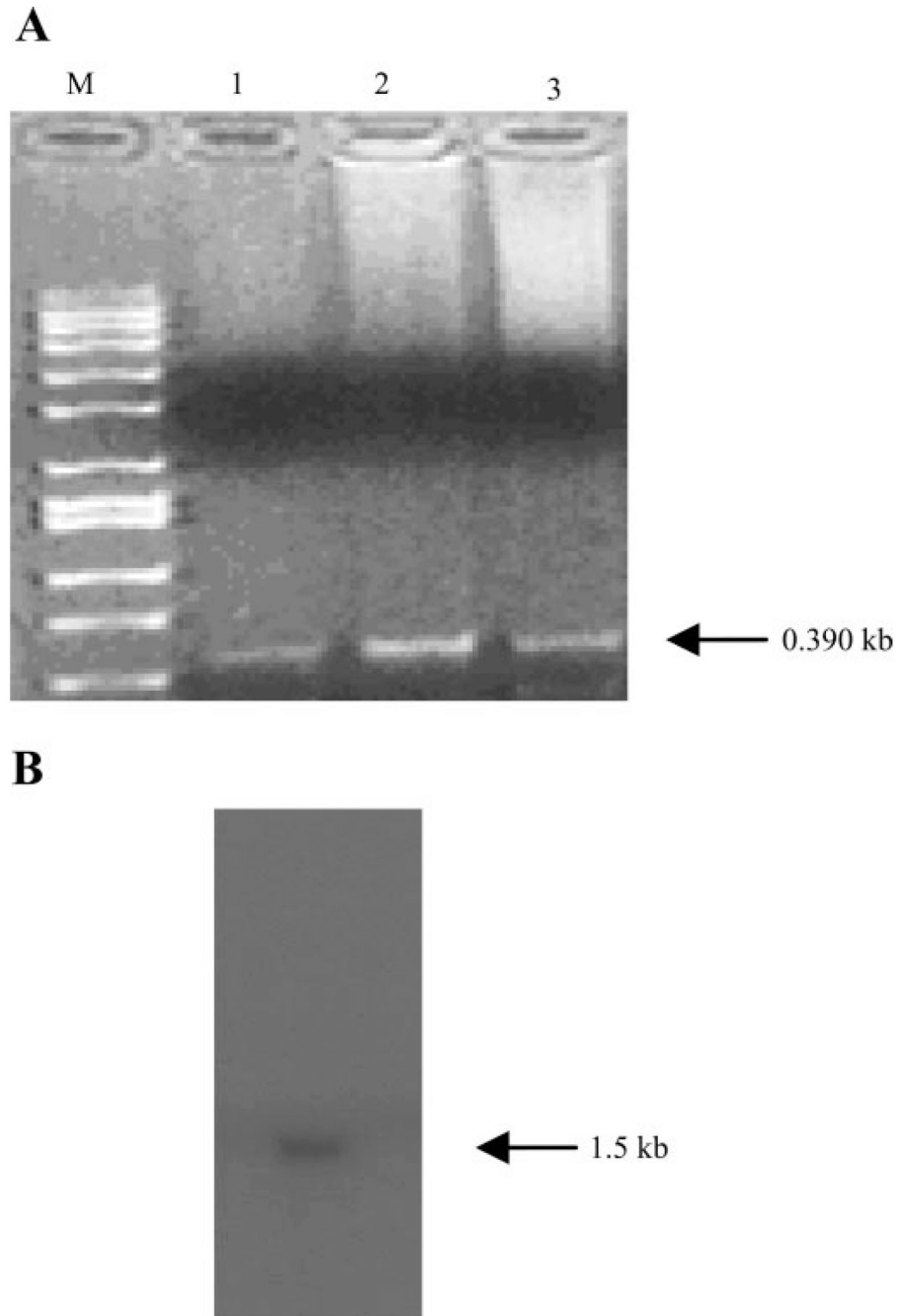


Fig. 3. Expression of IGFBP5-IP in various bone cell types. *A*: total RNA from human bone calvarial osteoblasts (HBC) (*lane 1*), MG63 (*lane 2*), and U2 (*lane 3*) human osteosarcoma cell lines was used for RT-PCR analysis using IGFBP5-IP-specific primers, as described in MATERIALS AND METHODS. IGFBP5-IP gene is expressed in all osteoblast cell lines tested as judged by the amplification of 0.390-kb RT-PCR product (CAF2/CAR285 primers) from HBC, MG63, and U2 cell lines. *B*: total RNA extracted from human U2 osteosarcoma cells was used to carry out Northern blot analysis using the IGFBP5-IP cDNA as probe, which detected a 1.5-kb band.

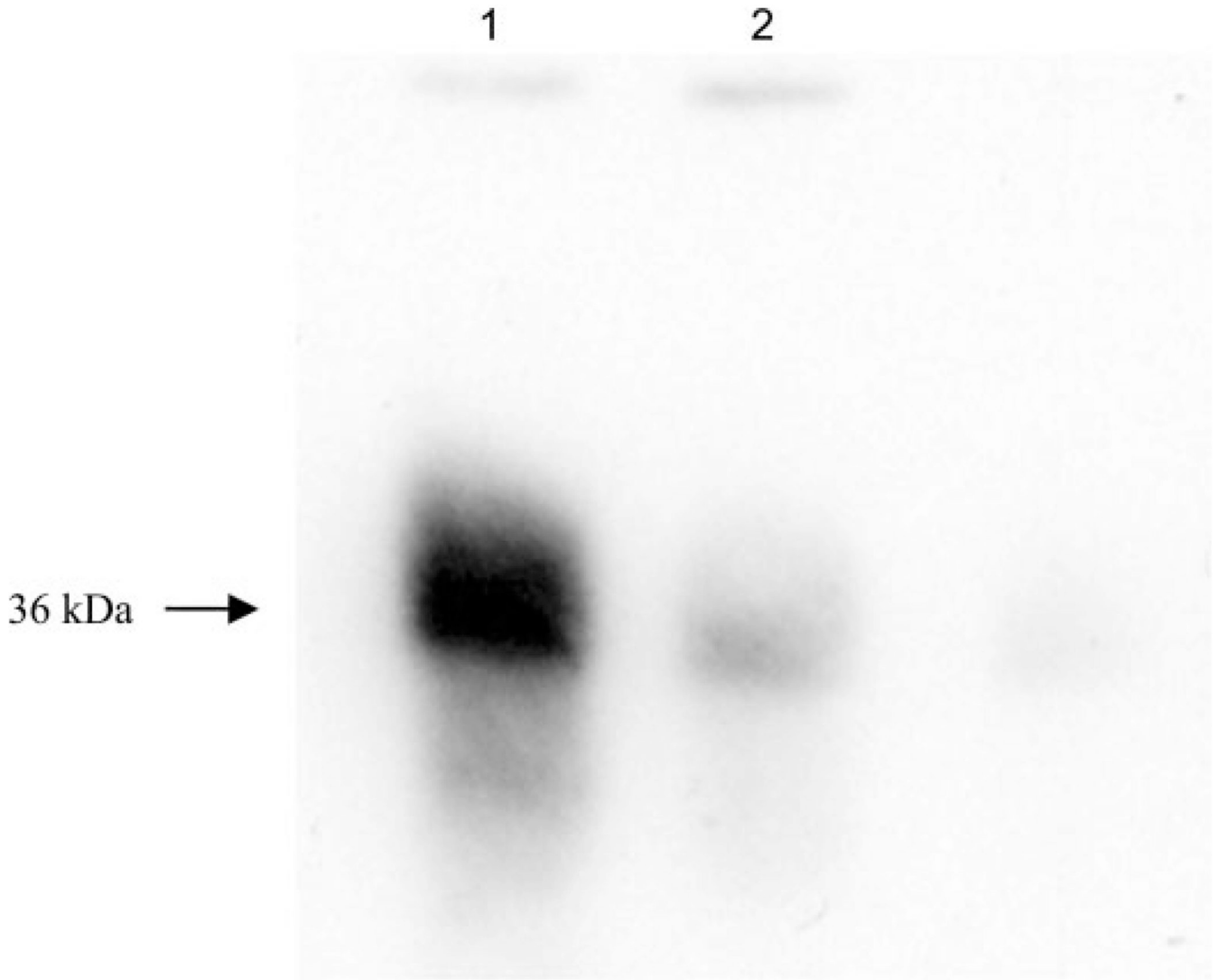


Fig. 4.

Coimmunoprecipitation of IGFBP5-IP/IGFBP5 in U2 cells overexpressing pFlag-IGFBP5-IP and IGFBP5. *Lane 1*, 250 μ l of cell lysate incubated with 25- μ l protein A sepharose conjugated to pFlag monoclonal antibody was incubated for 14 h at 4°C on a rotary shaker. The protein complex was then washed and subjected to SDS-PAGE and immunoblot analysis using IGFBP5 polyclonal antibody. *Lane 2* is the same as *lane 1*, except that protein A sepharose used was conjugated to mouse IgG antibody. IGFBP5-IP pulled down IGFBP5 present in the U2 cell lysate. Coimmunoprecipitation data together with the yeast two-hybrid system data provide evidence that IGFBP5-IP and IGFBP5 do interact.

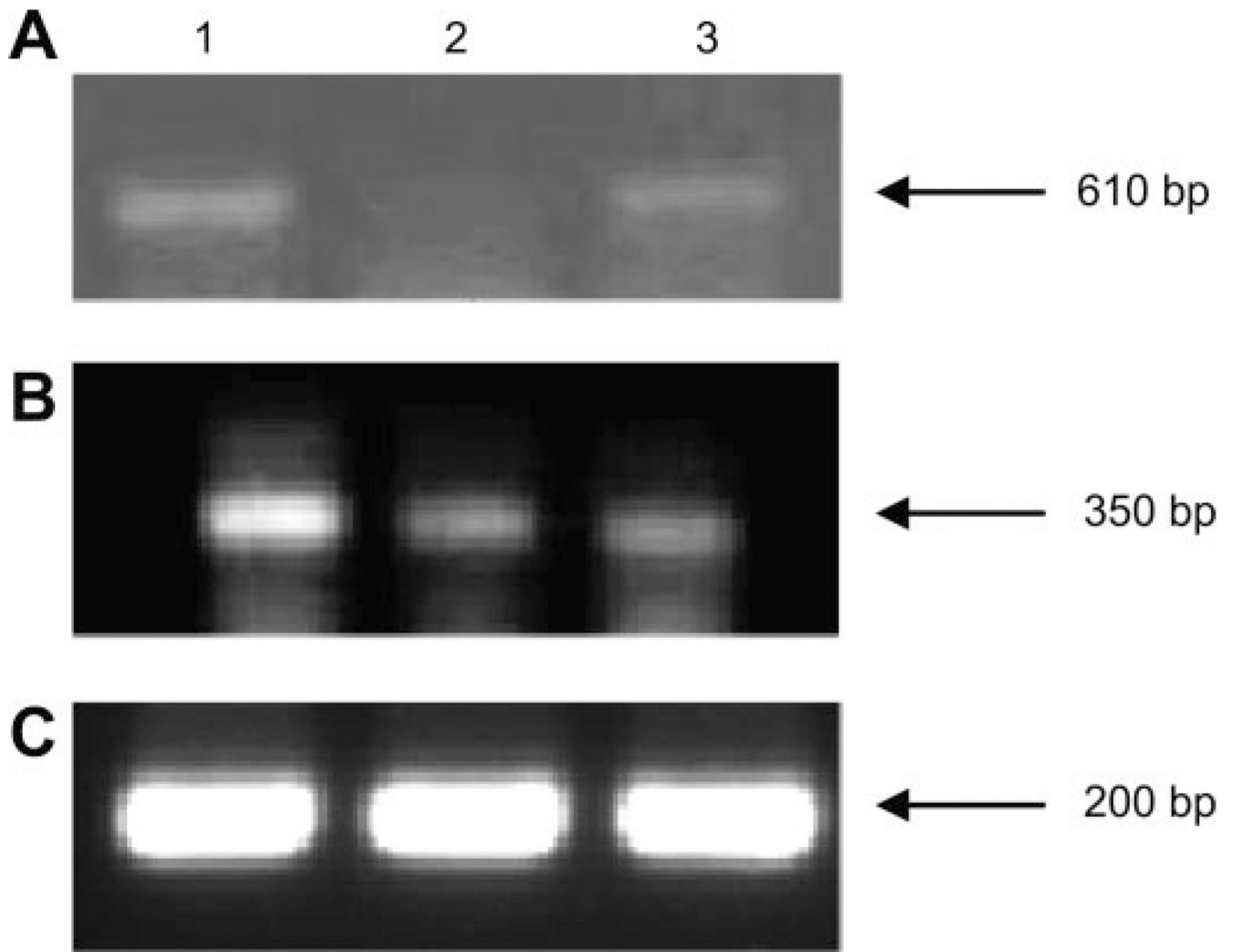


Fig. 5. Effects of pSuppressorNeo-IGFBP5-IP siRNA-1323 and siRNA-207 on mRNA levels of IGFBP5-IP and RIKEN cDNA clone in MC3T3-E1 cells. *Lane 1*, empty vector-treated control cells; *lane 2*, siRNA-1323-treated cells; and *lane 3*, siRNA-207-treated cells. The IGFBP5-IP-specific primers (pCAF2/pCAR1) gave a 610-bp product (*A*), RIKEN specific primers (pF596/pR618) gave a 350-bp product (*B*), and β -actin specific primers gave a 200-bp product (*C*). The siRNA-IGFBP5-IP encoded by pSuppressorNeo-IGFBP5-IP siRNA-1323 (Si-323) plasmid specifically targeted the degradation of IGFBP5-IP and RIKEN cDNA clone mRNAs as expected. Treatment of MC3T3-E1 with a hairpin form of siRNA-207 target specific for the RIKEN clone mRNA reduced the RIKEN clone mRNA but not IGFBP5-IP mRNA, suggesting that the RIKEN mRNA and IGFBP5-IP are two independent transcripts. Expression of actin was not altered by either siRNA-1323 or siRNA-207 treatment.

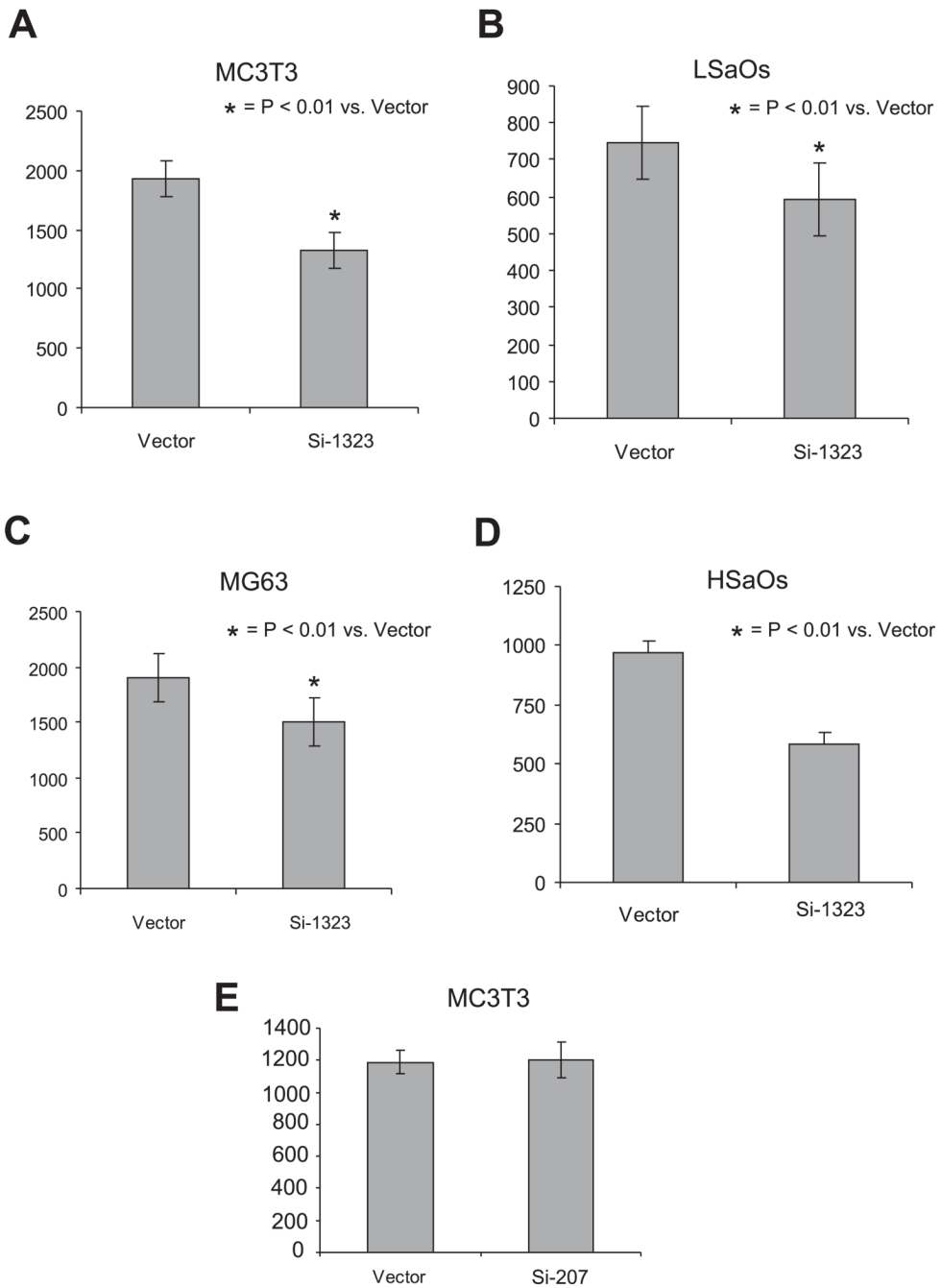


Fig. 6. Treatment with pSuppressorNeo-IGFBP5-IP siRNA-1323 (Si-1323) vector and pSuppressorNeo vector (control) and effects on cell number. MC3T3-E1 (A), LSaOs (B), MG63 (C), and HSaOs (D) cells were transfected with 1 μ g of plasmid DNA/ml using the effective reagent (Qiagen) and then incubated for 48 h before estimation of cell number by the AlamarBlue assay. Values for cell number are shown as arbitrary units in the y-axis and are means \pm SD of 8 replicates. * P < 0.05 vs. vector control. A hairpin form of siRNA-1323 specific for IGFBP5-IP mRNA resulted in a significant reduction in cell number compared with control in four different cell lines, thus suggesting a potential role for IGFBP5-IP in cell proliferation. Treatment of MC3T3-E1 cells with a hairpin form of siRNA-207 specific for the RIKEN clone

mRNA did not affect cell number compared with control (*E*), suggesting that silencing of RIKEN mRNA does not affect cell proliferation while silencing of IGFBP5-IP does.

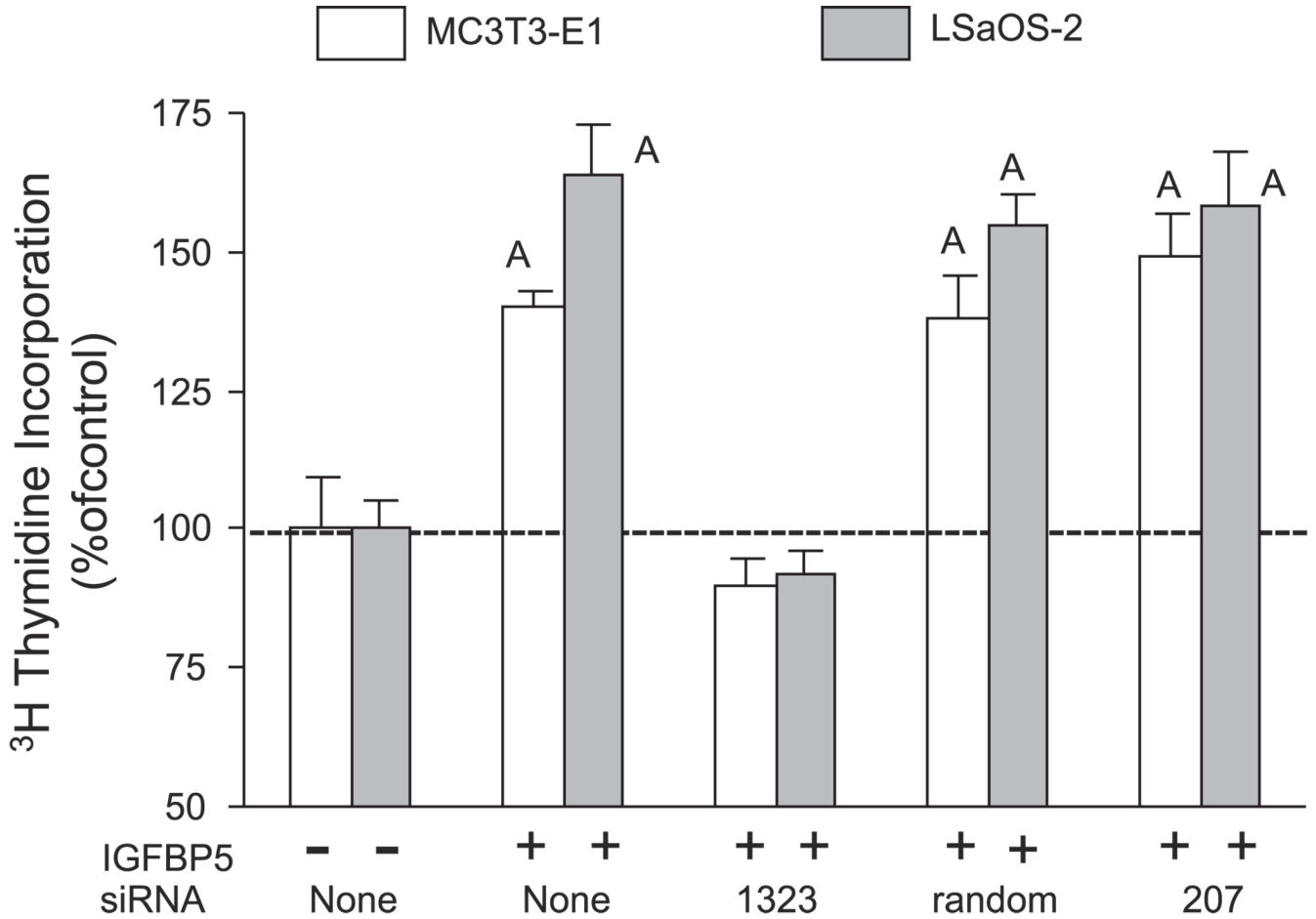


Fig. 7.

Effects of siRNA-1323, siRNA-207 and random control siRNA on IGFBP5-induced MC3T3-E1 (open bars) or LSAOs-2 cells (gray bars). Cells were incubated with transfection medium containing vehicle or siRNA, as described in MATERIALS AND METHODS. Twenty-four hours after transfection, the medium was changed to serum free, and the cells were incubated for another twenty-four hours before the addition of vehicle or 100 ng/ml IGFBP5. Eighteen hours later, ^3H -thymidine was added and labeled for six hours before termination of cultures. Values are means \pm SD of 8 replicates per group. The amount of ^3H -thymidine incorporated into control culture was 548 ± 49 and 1407 ± 10 cpm, respectively, in MC3T3-E1 and LSAOs cells. ^A $P < 0.05$ vs. control. siRNA-1323 blocked IGFBP5-induced increases in ^3H -thymidine incorporation.

Table 1

Specificity of interaction between clone A and IGFBP5, as judged by growth of reporter strain on high-stringency selection medium

Plasmid	pGBKT7-BP5	pGBKT7-lam (control)	pGBKT7-53 (control)	Clone Size
Clone A	+	-	-	0.8 kb
pGADT7-T (control)	-	-	+	NA

Cotransfection of the reporter yeast strain with the isolated clone A together with the BD bait plasmid activated all three reporter genes. +, the transformed AH109 strain grew on high-stringency medium; -, the transformed AH109 strain did not grow on high-stringency medium. NA, not applicable; IGFBP, insulin-like growth factor binding protein. The pGBKT7-BP5, -lam, and -53 encode fusions of Gal4 BD and human IGFBP5, human lamin C, and murine p53, respectively. The pGADT7 is a positive control that encodes a fusion of GAL4 AD and SV40 large T-antigen that is known to bind p53.