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IRS-1 Functions as a Molecular Scaffold to Coordinate IGF-I/IGFBP-2 Signaling During Osteoblast Differentiation

Gang Xi[1], **Xinchun Shen**[2], **Clifford J Rosen**[3], and **David R Clemmons**[1]

[1]Department of Medicine, University of North Carolina, School of Medicine, Chapel Hill, NC, USA

[2]College of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing, China

[3]Maine Medical Center Research Institute, Scarborough, ME, USA

Abstract

Insulin like growth factor I (IGF-I) and insulin like growth factor binding protein-2 (IGFBP-2) function coordinately to stimulate AKT and osteoblast differentiation. IGFBP-2 binding to receptor protein tyrosine phosphatase β (RPTPβ) stimulates polymerization and inactivation of phosphatase activity. Because phosphatase and tensin homolog (PTEN) is the primary target of RPTPβ, this leads to enhanced PTEN tyrosine phosphorylation and inactivation. However RPTPβ inactivation also requires IGF-I receptor activation. The current studies were undertaken to determine the mechanism by which IGF-I mediates changes in RPTPβ function in osteoblasts. IGFBP-2/IGF-I stimulated vimentin binding to RPTPβ and this was required for RPTPβ polymerization. Vimentin serine phosphorylation mediated its binding to RPTPβ and PKCζ was identified as the kinase that phosphorylated vimentin. To determine the mechanism underlying IGF-I stimulation of PKCζ-mediated vimentin phosphorylation, we focused on insulin receptor substrate–1 (IRS-1). IGF-I stimulated IRS-1 phosphorylation and recruitment of PKCζ and vimentin to phospho-IRS-1. IRS-1 immunoprecipitates containing PKCζ and vimentin were used to confirm that activated PKCζ directly phosphorylated vimentin. PKCζ does not contain a SH-2 domain that is required to bind to phospho-IRS-1. To determine the mechanism of PKCζ recruitment we analyzed the role of p62 (a PKCζ binding protein) that contains a SH2 domain. Exposure to differentiation medium plus IGF-I stimulated PKCζ/p62 association. Subsequent analysis showed the p62/PKCζ complex was co-recruited to IRS-1. Peptides that disrupted p62/PKCζ or p62/IRS-1 inhibited IGF-I/IGFBP-2 stimulated PKCζ activation, vimentin phosphorylation, PTEN tyrosine phosphorylation, AKT activation, and osteoblast differentiation. The importance of these signaling events for differentiation was confirmed in primary mouse calvarial osteoblasts. These results demonstrate the cooperative interaction between RPTPβ and the IGF-I receptor leading to a coordinated series of signaling events that are required for osteoblast differentiation. Our findings emphasize the important role IRS-1 plays in modulating these signaling events and confirm its essential role in facilitating osteoblast differentiation.

Address correspondence to: David R Clemmons, MD, CB7170, 8024 Burnett Womack, University of North Carolina, Chapel Hill, NC 27599-7170, USA. david_clemmons@med.unc.edu.

Disclosure

All authors state that they have no conflicts of interest.

Keywords

IRS-1; P62; PKCζ; VIMENTIN; OSTEOBLAST DIFFERENTIATION

Introduction

Insulin like growth factor I (IGF-I) and insulin like growth factor binding protein 2 (IGFBP-2) function coordinately to stimulate osteoblast differentiation.[1] IGF-I deletion in mice has been shown to result of reduction in bone volume and mineralization.[2–4] Deletion in the IGF-I receptor (IGF1R) resulted in smaller bones and deficient calcification.[5,6] Similarly, deletion of IGFBP-2 in male mice results in marked attenuation of bone volume/total volume in the distal femur and decreased mineralization.[7] Further studies showed that administration of a peptide containing the region of IGFBP-2 that bound to receptor protein tyrosine phosphatase β (RPTPβ), its cell surface receptor, rescued the phenotype in IGFBP-2$^{-/-}$ mice.[8] That this peptide contained the region of sequence within IGFBP-2 necessary for bioactivity was further demonstrated by showing that mutagenesis of this sequence within the intact protein resulted in an inability to stimulate osteoblast proliferation and/or differentiation. In addition, calvarial osteoblasts obtained from the IGFBP-2$^{-/-}$ mice showed attenuated differentiation, which could be rescued in the presence of either intact IGFBP-2 or the synthetic peptide containing the RPTPβ binding site.[1] However, exposure to this peptide or IGFBP-2 alone did not stimulate a significant increase in differentiation in vitro unless the cells were concomitantly stimulated with IGF-I, suggesting that these two peptides function coordinately.

Analysis of the signaling events that are stimulated by each peptide has shown that binding of each ligand to its receptor results in significant stimulation of the PI-3 kinase/AKT signaling pathway and inhibition of activation of either receptor results in attenuation in activation of this pathway as well as differentiation.[1] Following IGFBP-2 binding to RPTPβ, the receptor dimerizes, which in turn inhibits its tyrosine phosphatase activity.[9] A principal target of this phosphatase is phosphatase and tensin homolog (PTEN). Stimulation of RPTPβ polymerization results in increased tyrosine phosphorylation of PTEN that inhibits PTEN activity, thereby leading to enhanced AKT stimulation. Deletion of RPTPβ in osteoblasts leads to enhanced PTEN tyrosine phosphorylation and osteoblast differentiation.[1] However, our prior studies in vascular smooth muscle cells (VSMCs) showed that inhibition of RPTPβ polymerization required not only IGFBP-2 binding to RPTPβ but also IGF-I–stimulated IGF-I receptor tyrosine kinase activation.[9] In that cell type, stimulation of the RPTPβ polymerization was shown to require vimentin binding to the cytoplasmic domain of RPTPβ, and vimentin binding was mediated through vimentin serine phosphorylation.[10] Stimulation of the IGF-I receptor led to serine phosphorylation of vimentin and blocking either serine phosphorylation of vimentin or RPTPβ/vimentin association was shown to inhibit RPTPβ polymerization and AKT activation in response to IGF-I/IGFBP-2. However, that mechanism was only activated in the presence of high glucose concentrations: eg, 25 mM glucose.

Based on those observations the current studies were undertaken to determine whether vimentin binding to RPTPβ was stimulated by IGF-I in osteoblasts and whether this was required for RPTPβ polymerization. We also determined whether activation of the IGF-I receptor in the presence of normal glucose concentrations, eg, 5.6 mM, was sufficient to stimulate vimentin/RPTPβ association. Further studies analyzed the molecular mechanism by which IGF-I binding to the IGF-I receptor resulted in vimentin/RPTPβ association and the significance of these biochemical events for osteoblast differentiation.

Materials and Methods

Human IGF-I was a gift from Genentech (San Francisco, CA, USA). Immobilon-P membranes, amyristoylated form of cell-permeable PKCζ pseudosubstrate inhibitor (Cat#539624), protein A and G agarose were purchased from EMD-Millipore (Billerica, MA, USA). α-MEM, streptomycin, and penicillin were purchased from Life Technologies (Grand Island, NY, USA). PQ401 was purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against PTEN, phospho-vimentin (S39) phospho-AKT (S473), AKT, and pPKCζ (T410) were from Cell Signaling Technology Inc. (Beverly, MA, USA). The anti-phosphotyrosine (pY99), p62, PKCζ, fibronectin, and vimentin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An anti-phosphoserine (pSer) antibody was purchased from Abcam (Cambridge, MA, USA). An anti-RPTPβ monoclonal antibody was purchased from BD Bioscience (San Diego, CA, USA). SHPS-1 polyclonal antiserum was prepared as described.[11] The IGFBP-2 antibody was prepared as described.[7] The horseradish peroxidase–conjugated mouse anti-rabbit, goat anti-mouse, and mouse anti-rabbit light chain–specific antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Synthetic peptides containing the cell permeability sequence of protein transduction domain and the vimentin head domain sequence (underlined) (YARAAAR-QARARSVSSSSYRRMF), a control peptide (YARAAARQARARSVASAAYRRMF), a cell permeable peptide containing the underlined (PB-1 domain) sequence from p62 (YARAAARQARASLTVKAYLLGKE), and a control peptide (YARAAARQARAKEVYL-SLAGTLK), the cell-permeable peptides that contained Tyr-608 and Tyr-628, two of the three critical YXXM motifs in the IRS-1, (YARAAARQARATDDGYMPMSP and YARAAARQAR-AKGSGDYMPM), and a control peptide (YARAAARQAR-AMPMSPKSVSAP) were synthesized by the Protein Chemistry Core Facility at the University of North Carolina at Chapel Hill (Chapel Hill, NC, USA). Peptide purity and confirmation of the correct sequence were determined by mass spectrometry.

Cell culture

MC-3T3 E1 clone 4 (CL4) cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in α-MEM (glucose 1000 mg/L) containing 10% fetal bovine serum (Thermo-Fisher Scientific, Pittsburgh, PA, USA). After confluence, culture medium was changed to differentiation medium (DM) that contained 10% fetal bovine serum plus 50 μg/mL ascorbic acid and 4 mM β-glycerol phosphate. Fresh DM was applied every 72 hours. Each peptide

(10 μg/mL unless otherwise stated) was added with the differentiation medium and replaced every 72 hours.

All of the animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of University of North Carolina at Chapel Hill. Neonatal calvarial osteoblasts were isolated from 3-day-old to 5-day-old C57/B6J mice. Briefly, calvariae were digested five times with collagenase type 2 (250 unit/mL) and trypsin (0.05%) plus EDTA (0.02%) in the PBS. The cells released from digests 2 through 5 were collected as primary calvarial osteoblasts and maintained in α-MEM (glucose 1000 mg/L) supplemented with 10% FBS and nonessential amino acids.

Generation and purification of wild-type IGFBP-2 and an IGFBP-2 mutant

Wild-type mouse IGFBP-2 and middle heparin binding domain mutated IGFBP-2 (MT1) were generated and purified following the procedure as described.[9]

Construction of cDNAs and establishment of IGFBP-2 Si and LacZ Si cells

MC-3T3 cells expressing shRNA targeting IGFBP-2 and control shRNA were established following the procedures as described.[1]

Transient transfection with siRNA targeting vimentin

siRNA targeting vimentin (sc-29523) and a control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MC-3T3 cells were transfected using a concentration of 30 pM and the PepMute Plus reagent (SignaGen Laboratories, MD, USA) following the manufacturer's instructions. The experiments were initiated 72 hours after transfection.

Immunoprecipitation and immunoblotting

The cell monolayers were lysed in a modified radioimmunoprecipitation assay (RIPA) buffer as described.[1] Immunoprecipitation was performed by incubating 0.5 mg of cell lysate protein with 1 μg of each of the following antibodies: anti-PY99, anti-vimentin, anti-phospho-serine, anti-IRS, anti-p62, or anti-PKCζ at 4°C overnight. Immunoblotting was performed as described[1] using a dilution 1:1000 for anti-pAKT (Ser473), AKT, vimentin, vimentin (pSer39), PTEN, PKCζ, and p62 antibodies, a dilution 1:500 for anti-RPTPβ or anti-pPKCζ (Thr410), a dilution 1:150 for anti-osteocalcin, a dilution 1:5000 for anti-IRS-1 and β-actin and a dilution 1:10000 for anti-IGFBP-2. The proteins were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL, USA). Total cellular protein in the lysates was determined using bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, USA).

Chemical cross-linking

The chemical cross-linking procedures were performed as described.[10] Briefly, cells were washed three times with PBS, then incubated with 2 mg/mL bis[sulfosuccinimidyl]suberate, BS3 (Thermo Fisher Scientific, Rockford, IL, USA) in PBS for 1 hour on ice. Cross-linking was terminated by adding 50 mM Tris for 15 min. The cells were lysed and the lysate was separated using a 6% SDS-PAGE gel.

In vitro kinase assay

Nonphosphorylated or basal serine phosphorylated vimentin was prepared from quiescent osteoblasts before being exposed to differentiation medium by immunoprecipitating with an antivimentin antibody (1:500 dilution). The activated PKCζ that was associated with IRS-1 was prepared from osteoblasts cultured in differentiation medium for 3 days in the presence or absence of a PKCζ inhibitor. The complexes were immunoprecipitated from cell lysates using an anti-IRS-1 antibody (1:500 dilution). Control immune complexes were precipitated using lysates prepared from cells cultured using the same conditions in the absence of a PKCζ inhibitor and precipitated with a nonimmune IgG. To detect vimentin serine phosphorylation, vimentin immunoprecipitate (10 μg) was incubated with IRS-1 immunoprecipitate (10 μg) in 500 μL of kinase assay buffer (pH 7.5,25 mM Tris, 5 mM glycerophosphate, 1 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, and 40 μM ATP) for 30 min at 37°C. The proteins were separated using an 8% PAGE gel and immunoblotted with an anti-vimentin (pS39) antibody.

Alizarin Red staining

Cells were washed with PBS twice before were fixed with 10% formalin. After 10 min fixation, 1% Alizarin Red (pH 4.2) was applied and incubated for another 10 min before it was removed. Cells were washed with ddH_2O twice and drying. Images were captured using a Leica M420 microscope.

Statistical analysis

Densitometry results are expressed as the mean ± standard deviation (SD). All experiments were replicated at least three times to assure reproducibility. The results were analyzed for statistically significant differences using Student's *t* test or analysis of variance followed by Bonferroni multiple comparison post hoc test. Statistical significance was set at $p < 0.05$.

Results

To determine whether IGF-I receptor activation was required for IGFBP-2 to stimulate RPTPβ polymerization, PQ401, which inhibits IGF-I receptor tyrosine kinase activation, was utilized. Addition of IGF-I and IGFBP-2 to cultures exposed to differentiation medium resulted in stimulation of RPTPβ polymerization and addition of PQ401 inhibited polymerization (Fig. 1*A*). That activation of both receptors was required was demonstrated by adding an anti-fibronectin-3 antibody that inhibits binding of IGFBP-2 to RPTPβ and demonstrating that there was no stimulation of RPTPβ polymerization (Fig. 1*B*). The requirement of IGFBP-2 binding to RPTPβ was confirmed using IGFBP-2 siRNA, which resulted in inhibition of RPTPβ polymerization, which was restored with IGFBP-2 addition (4.0 ± 0.7 fold, $p = 0.001$) but not with an IGFBP-2 mutant which had had the RPTPβ binding site altered (Fig. 1*C*). Because our prior studies in VSMC had shown that vimentin binding to RPTPβ was required to stimulate RPTPβ polymerization, we utilized a cell permeable peptide that had been shown to disrupt their association. This peptide contains 11 amino acids located in the head domain of vimentin, a region that mediates the binding of vimentin to several proteins.[12] IGF-I stimulated vimentin/RPTPβ association 6.7 ± 1.0 fold ($p = 0.002$) and exposure to this peptide inhibited their association 79%±5% ($p < 0.001$)

(Fig. 1*D*). This was accompanied by a 77% ±12% ($p = 0.006$) reduction in RPTPβ polymerization (Fig. 1*E*). Additionally, IGFBP-2 binding to RPTPβ was also required to stimulate RPTPβ/vimentin association (Fig. 1*F*). To confirm the importance of vimentin binding to RPTPβ, we utilized RNAi to inhibit vimentin synthesis (Fig. 1*G*). Exposure of the cells to vimentin siRNA eliminated RPTPβ/vimentin association (Fig. 1*H*). This completely inhibited RPTPβ polymerization (Fig. 1*I*).

To determine the mechanism by which IGF-I receptor activation was stimulating vimentin binding to RPTPβ, we focused on vimentin serine phosphorylation because the sequence contained within the disrupting peptide contains three serine residues that are known to be phosphorylated. IGF-I stimulated vimentin serine phosphorylation that was reduced 84% ±11% ($p < 0.001$) following the addition of PQ401 (Fig. 2*A*). This resulted in loss of vimentin/RPTPβ association (Fig. 2*B*). In contrast, cells expressing IGFBP-2 siRNA showed no reduction in IGF-I-stimulated vimentin serine phosphorylation compared to control cultures, indicating that IGF-I receptor activation alone was sufficient (Fig. 2*C*). Because the disrupting peptide contained a region of sequence that is a consensus phosphorylation site for PKCζ,[13] we determined the effect of IGF-I on PKCζ activation. IGF-I stimulated an increase (3.8 ± 0.5 fold, $p = 0.003$) in PKCζ threonine 410 phosphorylation which is located in the autoactivation loop (Fig. 2*D*). Additionally IGF-I stimulated activated PKCζ/vimentin association (3.5 ± 0.3 fold, $p = 0.004$) (Fig. 2*E*). More importantly exposure to a specific PKCζ pseudosubstrate inhibitor inhibited IGF-I stimulated vimentin/RPTPβ association (Fig. 2*F*) vimentin serine phosphorylation (Fig. 2*G*) and the physiologically significant downstream event RPTPβ polymerization (Fig. 2*H*).

To determine the importance of these events for downstream signaling we analyzed the effect of inhibition of vimentin/RPTPβ association and PKCζ phosphorylation. As shown in Fig. 3*A*, the addition of the peptide that disrupted vimentin/RPTPβ association inhibited IGF-I-stimulated PTEN tyrosine phosphorylation and AKT activation (Fig. 3*A*). This finding was also confirmed using vimentin siRNA (Fig. 3*B*). Similarly, the addition of the PKCζ pseudosubstrate inhibitor reduced both PTEN tyrosine phosphorylation and AKT activation in response to IGF-I and IGFBP-2 (Fig. 3*C*, *D*).

To determine the mechanism by which activation of the IGF-I receptor facilitated PKCζ-mediated vimentin phosphorylation, we analyzed the importance of their recruitment to a specific signaling scaffold. Our prior studies in VSMC had shown that IGF-I stimulated recruitment of vimentin and PKCζ to the molecular scaffold, SHPS-1. Hence, we immunoprecipitated SHPS-1 and analyzed vimentin binding. SHPS-1 phosphorylation was barely detected in osteoblasts and there was no recruitment of vimentin (Supporting Fig. 1A). Furthermore, addition of a peptide that blocks the recruitment of SH2 domain-containing proteins to SHPS-1 resulted in no attenuation of vimentin serine phosphorylation (Supporting Fig. 1B). A well-characterized target of the IGF-I receptor kinase is the scaffolding protein IRS-1, which is expressed in preosteoblasts and is required for normal osteoblast differentiation.[14] IRS-1 knockout mice have been shown to have significant reduction in bone volume and bone mineral density.[15] Therefore, we determined whether

Additional Supporting Information may be found in the online version of this article.

IRS-1 was functioning as the scaffold for recruiting vimentin and PKCζ in response to IGF-I receptor stimulation. IGF-I addition to osteoblast cultures stimulated a marked increase in IRS-1 tyrosine phosphorylation (Fig. 4*A*) and IRS-1/vimentin association (Fig. 4*B*). Importantly, immunoprecipitation of IRS-1 after IGF-I stimulation also resulted in coprecipitation of vimentin that was phosphorylated on serine 39, a known PKCζ phosphorylation site (Fig. 4*C*). In contrast, analysis of the supernatant that was not immunoprecipitated showed no vimentin serine 39 phosphorylation (Fig. 4*C*). This further supported the conclusion that PKCζ was the kinase that was phosphorylating vimentin and that it was recruited to IRS-1 in response to IGF-I stimulation. Next we immunoprecipitated IRS-1 and immunoblotted for PKCζ. IGF-I stimulated an increase in IRS-1–associated PKCζ and the PKCζ that was immunoprecipitated was activated (Fig. 4*D*). To confirm that PKCζ was the kinase that phosphorylated vimentin, we immunoprecipitated IRS-1 that contained activated PKCζ in the complex from cultures that had been stimulated to differentiate and vimentin was also pulled down from the nondifferentiating cultures. The two immunoprecipitates were analyzed for kinase activity in vitro and vimentin serine 39 phosphorylation was determined. As shown in Fig. 4*E*, vimentin serine 39 phosphorylation was significantly increased (3.7 ± 0.9 fold, $p = 0.009$) when the IRS-1 immunocomplex was present compared to normal IgG immunocomplex. Importantly, increased vimentin phosphorylation was prevented when IRS-1 was immunoprecipitated from cultures that had been exposed to a PKCζ inhibitor. To confirm this result in cells, we utilized the PKCζ pseudosubstrate inhibitor. Addition of this inhibitor also inhibited serine phosphorylation of vimentin that was associated with IRS-1 (Fig. 4*F*).

To confirm that phosphorylation was occurring on the IRS-1 scaffold we determined the mechanism of recruitment of PKCζ to IRS-1 in response to IGF-I. Following tyrosine phosphorylation of IRS-1 several SH2 domain containing proteins are recruited through SH2 domain-phosphotyrosine interactions. However, PKCζ does not contain a SH2 domain, therefore we determined if a protein which is known to interact with PKCζ that contained an SH2 domain could mediate its translocation to IRS-1. P62, sequesterosome-1, is an SH2 domain containing protein that interacts with PKCζ through its PB-1 domain and this interaction has been shown to be stimulated by IGF-I.[16] Additionally, p62 has been shown has been shown to be recruited to IRS-1.[17] Exposure of osteoblasts to IGF-I resulted in an increase in p62/PKCζ association (Fig. 5*A*). Because this interaction occurs through the PB-1 domain in p62 we utilized a cell-permeable peptide containing the PB-1 domain sequence to disrupt the p62/PKCζ interaction. The peptide disrupted p62/PKCζ association following IGF-I stimulation (Fig. 5*A*), but more importantly it inhibited the recruitment of PKCζ to IRS-1 (Fig. 5*B*) and PKCζ activation (Fig. 5*C*). This led to attenuated vimentin serine phosphorylation (Fig. 5*D*) and RPTPβ polymerization (Fig. 5*E*) in response to IGF-I. Consequently, exposure to this peptide inhibited IGF-I–stimulated PTEN tyrosine phosphorylation and AKT activation (Fig. 5*F*). To confirm that p62 was mediating PKCζ transfer, we utilized cell-permeable peptides which contained two of the three critical YXXM motifs that are located within an IRS-1, that mediate the binding to SH2 domain containing proteins. These tyrosines are directly phosphorylated by the IGF-I receptor.[18] Utilizing cell-permeable peptides containing the sequences flanking tyrosine 608 and 628, we demonstrated that inhibition of binding to these phosphotyrosines resulted in an

attenuation of recruitment of p62 to IRS-1 (Fig. 6*A*). Importantly, blocking p62 recruitment completely inhibited PKCζ recruitment to IRS-1 (Fig. 6*B*) and this significantly attenuated PKCζ activation (Fig. 6*C*) and vimentin serine phosphorylation (Fig. 6*D*).

Insulin is also an important stimulant of IRS-1 phosphorylation and osteoblast differentiation.[19] The addition of a concentration of insulin (1×10^{-9} M) that stimulates the insulin but not the IGF-I receptor was as effective as IGF-I in stimulating p62 translocation to IRS-1; however, it did not result in maximal PKCζ activation (2.0 ± 0.2 fold versus 4.1 ± 0.5 fold increase, $p = 0.001$) or vimentin serine 39 phosphorylation (2.2 ± 0.3 fold versus 4.4 ± 0.8 fold increase, $p = 0.009$) (Supporting Fig. 2A, B). In contrast, 1×10^{-7} M insulin (a concentration that activates the IGF-I receptor) stimulated a significant increase in PKCζ activation (3.8 ± 0.2 fold increase, $p = 0.001$). To determine if this difference was physiologically relevant we analyzed osteoblast differentiation. The addition of 1×10^{-9} M insulin stimulated osteoblast differentiation, but the response was clearly less than the response to IGF-I. In contrast, 1×10^{-7} M insulin, which activates the IGF receptor, resulted in a greater response (Supporting Fig. 2C).

To determine the significance of activation of this signaling cascade for osteoblast differentiation, we utilized the peptide that disrupted vimentin/RPTPβ association, vimentin knockdown, the peptide that disrupted p62/PKCζ association, and the peptide that inhibited p62 transfer to IRS-1. Addition of each of the three peptides or knockdown of vimentin resulted in major attenuation of the ability of IGF-I/IGFBP-2 to stimulate osteoblast differentiation as well was osteocalcin expression (Fig. 7*A*–*D*). These peptides had no effect on cell viability (Supporting Fig. 3).

To determine the importance of these findings for primary osteoblast differentiation we analyzed changes in primary calvarial osteoblasts derived from C57BL6J newborn mice. Stimulation of these cultures with IGF-I showed that it stimulated RPTP/vimentin association and inhibition of this interaction resulted in attenuation of PTEN tyrosine phosphorylation and AKT activation (Fig. 8*A*). Addition of the PKCζ pseudosubstrate inhibitor blocked vimentin serine S39 phosphorylation (71% ± 3% reduction, $p < 0.001$) and vimentin/RPTPβ association (62% ±3% reduction, $p < 0.001$) (Fig. 8*B*). We also utilized the cell-permeable peptides and determined that IGF-I stimulated the recruitment of p62 to IRS-1, PKCζ activation and vimentin serine 39 phosphorylation were inhibited (77% ±11% reduction, $p < 0.001$; 69% ±15% reduction, $p = 0.002$, and 71% ±19% reduction, $p = 0.019$, respectively) (Fig. 8*C*). Importantly, inhibition of vimentin/RPTPβ association, PKCζ activation, or recruitment of p62 to IRS-1 inhibited osteoblast differentiation (Fig. 8*D*–*F*). Therefore, it appears that the major findings delineated in MC-3T3 cells were also reproduced in neonatal calvarial osteoblasts. Addition of the peptides did not stimulate apoptosis (Supporting Fig. 4).

In summary, IGF-I receptor mediated phosphorylation of tyrosines contained within YXXM motifs in IRS-1 results in recruitment of p62. Exposure to IGF-I stimulates p62/PKCζ association and the PKCζ that associates with p62 is then co-recruited to IRS-1 where it phosphorylates vimentin. This results in vimentin binding to RPTPβ, RPTPβ polymerization, and optimal activation of the AKT signaling pathway (Fig. 9). This

coordinated series of signaling events is required for cooperativity between RPTPβ and the IGF-I receptor leading to optimal stimulation of osteoblast differentiation.

Discussion

The major finding in this study is that after activation of the IGF-I receptor, a signaling complex is assembled on the scaffolding protein IRS-1 and this is required for IGFBP-2 to stimulate RPTPβ polymerization and osteoblast differentiation. Our prior studies showed that IGFBP-2 binding to RPTPβ played a major regulatory role in osteoblast differentiation by inhibiting PTEN activity, thereby leading to enhanced AKT signaling in response to IGF-I.[1] However, in addition to IGFBP-2 binding to RPTPβ, IGF-I receptor activation was absolutely required for PTEN inactivation. Specifically, when we overexpressed IGFBP-2 there was increased PTEN tyrosine phosphorylation and the number of cells that differentiated was stimulated, but if a IGF-I receptor tyrosine kinase inhibitor was added, IGFBP-2 binding to RPTPβ did not attenuate PTEN activity or stimulate differentiation. This suggested that IGF-I receptor activation was functioning coordinately with IGFBP-2 to stimulate RPTPβ polymerization and inactivation. These studies were undertaken to determine the signaling events that were stimulated by IGF-I which were required for IGFBP-2 to stimulate RPTPβ polymerization. The results clearly show that IGF-I stimulates vimentin binding to RPTPβ and that disruption of vimentin/RPTPβ association inhibits RPTPβ polymerization even in the presence of maximum IGFBP-2 concentrations. This was confirmed using vimentin knockdown. To determine the change that led to vimentin binding to RPTPβ we evaluated vimentin serine phosphorylation because it had been shown to mediate vimentin/RPTPβ association.[10,12] IGF-IR activation stimulated vimentin serine phosphorylation and this effect was shown to be mediated through PKCζ.

To analyze the mechanism by which stimulation of IGF-IR led to PKCζ-mediated vimentin phosphorylation, we determined if vimentin and PKCζ were co-recruited to the scaffolding protein IRS-1, which is directly phosphorylated by IGF-IR.[20] Importantly, our results showed that PKCζ and vimentin were recruited to IRS-1 and that IRS-1 associated PKCζ directly phosphorylated vimentin. Additional experiments showed that PKCζ was recruited to IRS-1 indirectly and that this effect was mediated by p62, a protein which had been shown to bind to PKCζ[21] and to be recruited to IRS-1 in response to insulin receptor stimulation.[17] IGF-I stimulated p62/PKCζ association and p62 bound directly to phosphotyrosines on IRS-1 that are known to be phosphorylated by the IGF-I receptor.[18] That PKCζ was co-recruited to IRS-1 with p62 was confirmed by disrupting p62/PKCζ or p62/IRS-1 and showing that this blocked PKCζ recruitment and activation. The importance of IGF-IR–mediated IRS-1 phosphorylation was further demonstrated using a phosphotyrosine blocking peptide that inhibited p62 binding to the phosphotyrosines on IRS-1 and showing that this inhibited p62/PKCζ recruitment and vimentin phosphorylation. Therefore, our studies define a unique signaling mechanism by which the IGF-I receptor phosphorylates IRS-1, allowing it to function as a molecular scaffold for assembly of a complex that is necessary to enable IGF-I to stimulate vimentin binding to RPTPβ (Fig. 9). Because this event is critical for regulating PTEN activity and osteoblast differentiation, the results provide a new understanding of the level of molecular control by which IGF-I and its

cognate receptor function to coordinate downstream signaling that is modulated by changes in RPTPβ activation.

In our studies, disruption of vimentin binding to RPTPβ resulted in an inability of IGFBP-2 to stimulate RPTPβ polymerization and an inhibition of downstream signaling. That this interaction required IGF-I receptor activation was proven by showing that inhibition of IGF-IR tyrosine kinase inhibited vimentin binding. IGF-I has been shown to stimulate vimentin rearrangement in a variety of cell types.[22] Although stimulating vimentin rearrangement could be related to its interaction with RPTPβ, our studies clearly show that serine phosphorylation of the vimentin head domain is required. Serine phosphorylation of the head domain has been shown to regulate differential changes in vimentin-protein interactions and changes in target protein function.[23,24] Vimentin synthesis is upregulated early in osteoblast differentiation.[25] Of 588 genes analyzed vimentin was one of 10 that showed the greatest increase during differentiation. Analysis following retinoic acid stimulation showed that it was one of six mRNAs that showed the greatest increase, and this change was accompanied by increased differentiation.[26] PTH-mediated changes in intracellular calcium lead to increased vimentin synthesis and changes in cytoskeletal assembly in osteoblasts.[27] Taken together with our results we conclude that induction of vimentin phosphorylation early in differentiation facilitates RPTPβ inactivation, and this may be one of the multiple functions of this protein that lead to changes which are essential for activation of the PI-3 kinase pathway that is required for differentiation to progress.

To link the increase in vimentin phosphorylation to IGF-IR stimulation we focused on the serine threonine kinase PKCζ for several reasons. First, the head domain of vimentin that was phosphorylated contains several PKCζ consensus phosphorylation sequences.[13] Second, PKCζ phosphorylates vimentin and this process is stimulated by IGF-I, although in VSMCs this occurred only in the presence of hyperglycemia.[10] Third, IGF-I has been shown to induce PKCζ activation.[28] That PKCζ was the kinase that mediated vimentin phosphorylation was shown by using a specific PKCζ pseudosubstrate inhibitor which functions in part by inhibiting the ability of PKCζ to be activated through binding to chaperone proteins.[29] Importantly, this pseudosubstrate inhibitor inhibited vimentin phosphorylation and RPTPβ polymerization as well as downstream signaling in response to IGF-I/IGFBP-2. In vitro studies have shown that PKCζ activation occurs during osteoblast differentiation.[30] Geng and colleagues[31] showed a low level of expression of PKCζ in proliferating osteoblasts, but there was a major increase in protein expression in high-density postconflict cultures. Fluid shear stress enhances IGF-I receptor–linked signaling in MC-3T3 cells; this change is PKCζ-dependent and functions to inhibit apoptosis.[32] Zini and colleagues[33] analyzed dysfunctional osteoblasts from patients with osteoarthritis and demonstrated a marked reduction in PKCζ expression and activation compared to posttraumatic osteoblasts isolated from normal subjects. Osteoblasts from arthritic patients had reduced expression of PKCζ due to induction of IL-1β or TNF-α, suggesting that the stress stimuli which lead to decreased bone mineral density could be functioning in part through this mechanism.[34] Our findings suggest that under normal differentiation conditions PKCζ may be utilized to activate AKT signaling through coordinated IGF-I receptor/RPTPβ activation and that cytokines which downregulate this enzyme could inhibit osteoblast differentiation through this mechanism.

Regulation of PKCζ subcellular localization is an important regulatory variable for determining substrate specificity. We had shown that PKCζ and vimentin were co-recruited to the scaffolding protein SHPS-1 in VSMCs.[10] However, when we analyzed SHPS-1 in osteoblasts there was minimal change in tyrosine phosphorylation in response to IGF-I and no recruitment of PKCζ or vimentin. Unlike VSMCs, osteoblasts maintained under normoglycemic conditions express a high concentration of the scaffolding protein IRS-1. IRS-1 is directly phosphorylated on three tyrosines contained in YXXM motifs by the IGF-I receptor and these phosphotyrosines recruit SH2 domain containing proteins such as the p85 subunit of PI-3 kinase. However, PKCζ does not contain a SH2 domain. Therefore, we investigated known PKCζ binding proteins that contain SH2 domains. p62, sequestosome, has been shown to associate with PKCζ after cytokine stimulation.[21] Our results showed that PKCζ is bound to p62 following the addition of IGF-I. Importantly, p62 was translocated to IRS-1 following IGF-I stimulation and blocking PKCζ/p62 association inhibited PKCζ transfer. Additionally, blocking p62 binding to IRS-1 also decreased PKCζ transfer and inhibited the ability of PKCζ to stimulate vimentin phosphorylation. Therefore, p62 is an important mediator facilitating PKCζ and vimentin colocalization on IRS-1.

A role for p62 in osteoblast function has not been previously reported. p62 mutations induce osteoclast activation in Paget's disease and osteoblast function has been shown to be altered in patients with this disorder.[35] Our findings define a role for p62 in osteoblast differentiation and suggest that it may be useful to analyze the effect of p62 and PKCζ, specifically in osteoblasts in patients with Paget's disease because p62 has been shown to be an important regulator of osteoclast function during the development of Pagetic bone changes.[36]

Our results emphasize the important functional role of IRS-1 in mediating the effects of IGF-I on osteoblast differentiation. Knockdown of IRS-1 in mice leads to a distinct bone phenotype that is associated with decreased bone turnover[15] and impaired fracture healing after injury.[37] IRS-$1^{-/-}$ mice have severe osteopenia, reduced trabecular and cortical thickness, and decreased bone formation rate.[15] Their calvarial osteoblasts had reduced induction of alkaline phosphatase by insulin or IGF-I. Consistent with these findings Granero-Molto and colleagues[38] showed impaired fracture healing and reduced bone mineral content within the fracture site in IRS-1–deficient mice. Injection of mesenchymal stem cells (MSCs) from wild-type mice that expressed IGF-I directly into the fracture site resulted in restoration of callus formation and union, indicating these cells could rescue the phenotype induced by IRS-1 deficiency. Analysis of a spontaneous mutant with stunted growth showed that those mice expressed a form of IRS-1 which contained only 15% of the intact sequence.[14] Those animals expressing the mutant form of IRS-1 had low bone mineral density, reduced trabecular and cortical thickness, and low bone formation rates. Osteoblast number was normal but alkaline phosphatase staining was reduced, and bone formation rate was suppressed, suggesting that the primary defect in these mice was failure of the osteoblasts to differentiate fully.

Studies using bone anabolic factors such as PTH which stimulate autocrine signaling by IGF-I through the IGF-I receptor have shown that IRS-1 expression is required for a normal bone anabolic response to PTH.[39] IRS-1 knockout mice had blunted stimulation of bone

turnover after 4 weeks of PTH compared to controls. Knockdown of IRS-1 using RNA interference in cultured osteoblasts leads to impaired collagen-1 synthesis and differentiation.[40–42] Additionally, IRS-1 expression is impaired in a variety of pathophysiologic processes that are associated with decreased osteoblast function.[6,43,44] Therefore, it appears that expression of adequate levels of IRS-1 and maintenance of the ability of IGF-I and insulin to stimulate IRS-1 tyrosine phosphorylation is required for normal preosteoblast differentiation. Our results suggest that insulin may function to stimulate osteoblast differentiation in part through stimulation of this pathway through IRS-1 phosphorylation; however, optimal PKCζ activation requires IGF-I receptor stimulation. Taken together with our findings it appears that several early signaling events in differentiation which require IGF-I stimulation are occurring on this signaling scaffold; therefore, stimulation of tyrosine phosphorylation of IRS-1 is critical for osteoblast differentiation.

Our results show that IGF-I is functioning through IGF-IR and IRS-1 to regulate vimentin phosphorylation, leading to subsequent IGFBP-2–stimulated RPTPβ polymerization and enhanced osteoblast differentiation. Prior studies showed that knockdown of the IGF-I receptor impaired the rate of bone formation in mice.[5] A conditional knockout demonstrated that bone mineral density was markedly lower at age 3 months compared to wild-type littermates of the same age and that trabecular volume and thickness were reduced.[43] Histomorphometry showed a significant reduction in bone formation rate and a clear defect in differentiation.[3,43] Knockdown of IGF-IR also results in a major reduction in the rate of mineral apposition and diminished bone mineral density.[5] Prior studies have emphasized defects in AKT and mammalian target of rapamycin (mTOR) activation, both of which are downstream from and modulated by changes in RPTPβ activation.[3,6] The results of this study complement those reports by showing that these receptors function cooperatively to stimulate osteoblast differentiation and define the early signaling events which are required following IGF receptor stimulation in order for IGFBP-2 to activate RPTPβ. The results emphasize the importance of coordinate signaling that does not involve the binding of IGF-I to IGFBP-2, but rather induction of signaling through these two pathways, and emphasize the importance of simultaneous increases in ligand occupancy of the respective receptors to fully augment AKT activation and thereby optimizing differentiation. Our in vitro studies show that loss of signaling through either pathway results in a major reduction in osteoblast differentiation.[1] Analysis of these pathways in vivo shows significant but less attenuation osteoblast activity, supporting the conclusion that compensatory mechanisms may be activated.[3,7,8] IGFBP-2$^{-/-}$ mice show increases in serum IGF-I and insulin, suggesting that this may be one mechanism by which compensation occurs. Future studies will be required to determine how these compensatory changes occur and how coordinate regulation of these pathways influences the rates of bone formation and mineral apposition that are IGF-IR–dependent.

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Authors' roles: Study design: GX, CJR, and DRC; Data collection: GX and XCS; Data analysis and interpretation: GX, XCS, CJR, and DRC; Drafting and reviewing manuscript: GX, CJR, and DRC.

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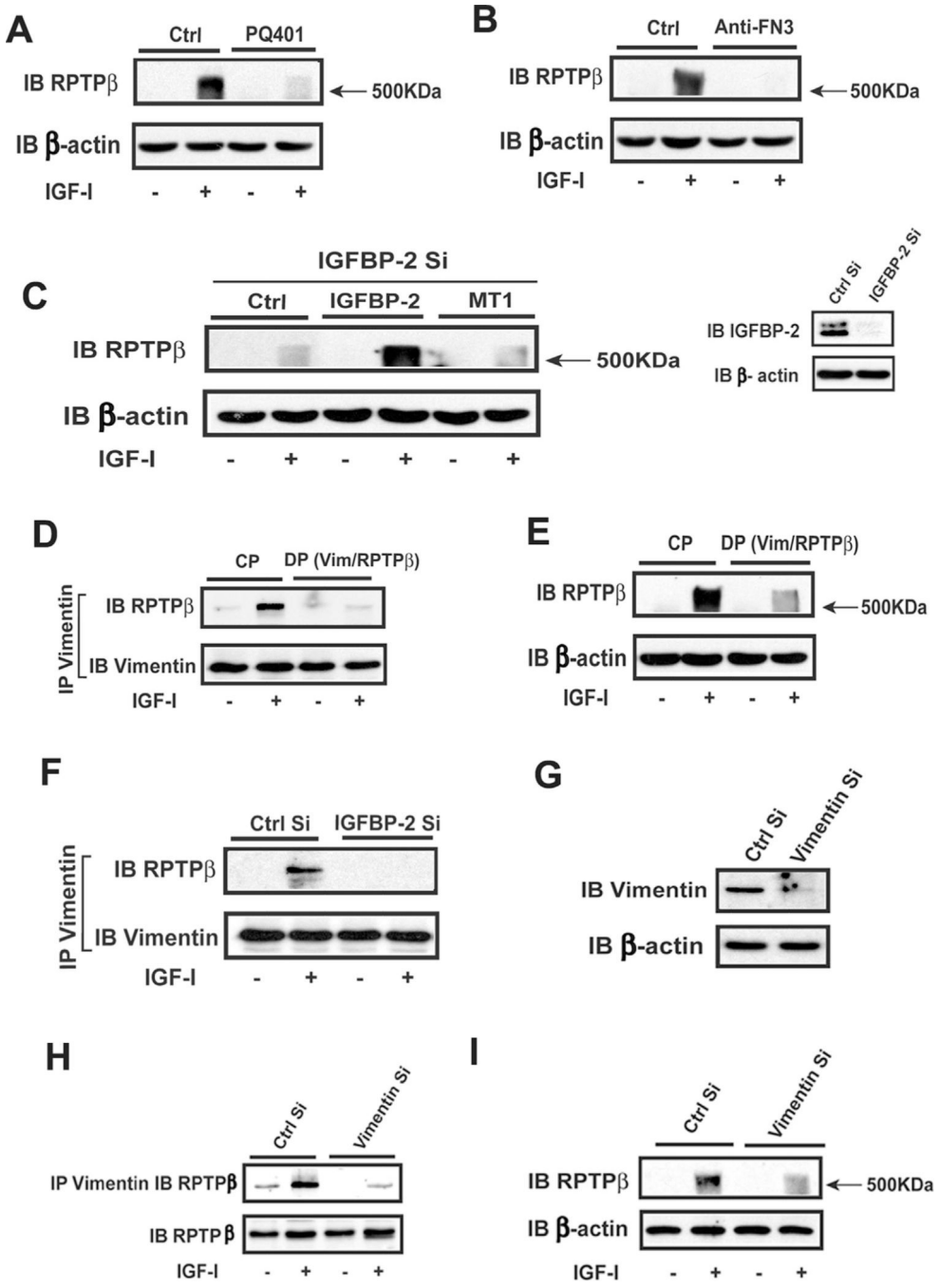

Fig. 1.

IGF-I and IGFBP-2 cooperatively stimulate vimentin/RPTPβ association and RPTPβ polymerization. (*A, B, E, I*) Cell lysates were prepared from quiescent MC-3T3 cells that had been exposed to differentiation medium for 3 days and incubated with (+) or without (–) IGF-I (100 ng/mL) for 5 min. The non-cell-permeable cross-linker bis[sulfosuccinimidyl]suberate (BS3) (+) was added as described in Materials and Methods. Two hours before harvesting, cells were either incubated with (*A*) PQ401 (25 μM) or DMSO (Ctrl) (*B*) anti-fibronectin (anti-FN3) antibody (1 μg/mL) or normal IgG (Ctrl) (*E*) a

vimentin/RPTPβ DP (10 μg/mL) or CP. (*I*) Cells were treated with a control siRNA (Ctrl si) or vimentin siRNA (Vimentin Si) (30 pM) for 72 hours. The cell lysates were separated using 6% SDS-PAGE, and immunoblotted with an anti-RPTPβ or β-actin antibody. (*C*) MC-3T3 cells expressing IGFBP-2 shRNA then treated with (+) or without (–) IGF-I for 5 min were incubated with wild-type IGFBP-2 or the middle heparin domain mutant (MT1) (200 ng/mL) or without protein (Ctrl) for 4 hours at 4°C, the cross-linker was added, and the cell lysates were analyzed as for *A*. Equal amounts of cell lysate from MC-3T3 cells expressing a shRNA sequence targeting LacZ (Ctrl Si) or IGFBP-2 (IGFBP-2 Si) were immunoblotted with indicated antibody. (*D*, *F*) MC-3T3 cells were prepared as in *A* then incubated with a CP or a vimentin/RPTPβ DP for 2 hours (*D*) or they were expressing control shRNA (Ctrl Si) or IGFBP-2 shRNA (IGFBP-2 Si) (*F*). Cell lysates were immunoprecipitated with an anti-vimentin antibody and immunoblotted with an anti-RPTPβ or anti-vimentin antibody. (*G*, *H*) MC-3T3 cells were treated with a control siRNA (Ctrl Si) or vimentin siRNA (vimentin Si) for 72 hours. They were immunoblotted with an anti-vimentin or β-actin antibody (*G*) or immunoprecipitated with an anti-vimentin antibody and immunoblotted with an anti-RPTPβ antibody (*H*). The same amount of lysate was immunoblotted with an anti-RPTPβ antibody (*H*). CP = control peptide; DP = disrupting peptide.

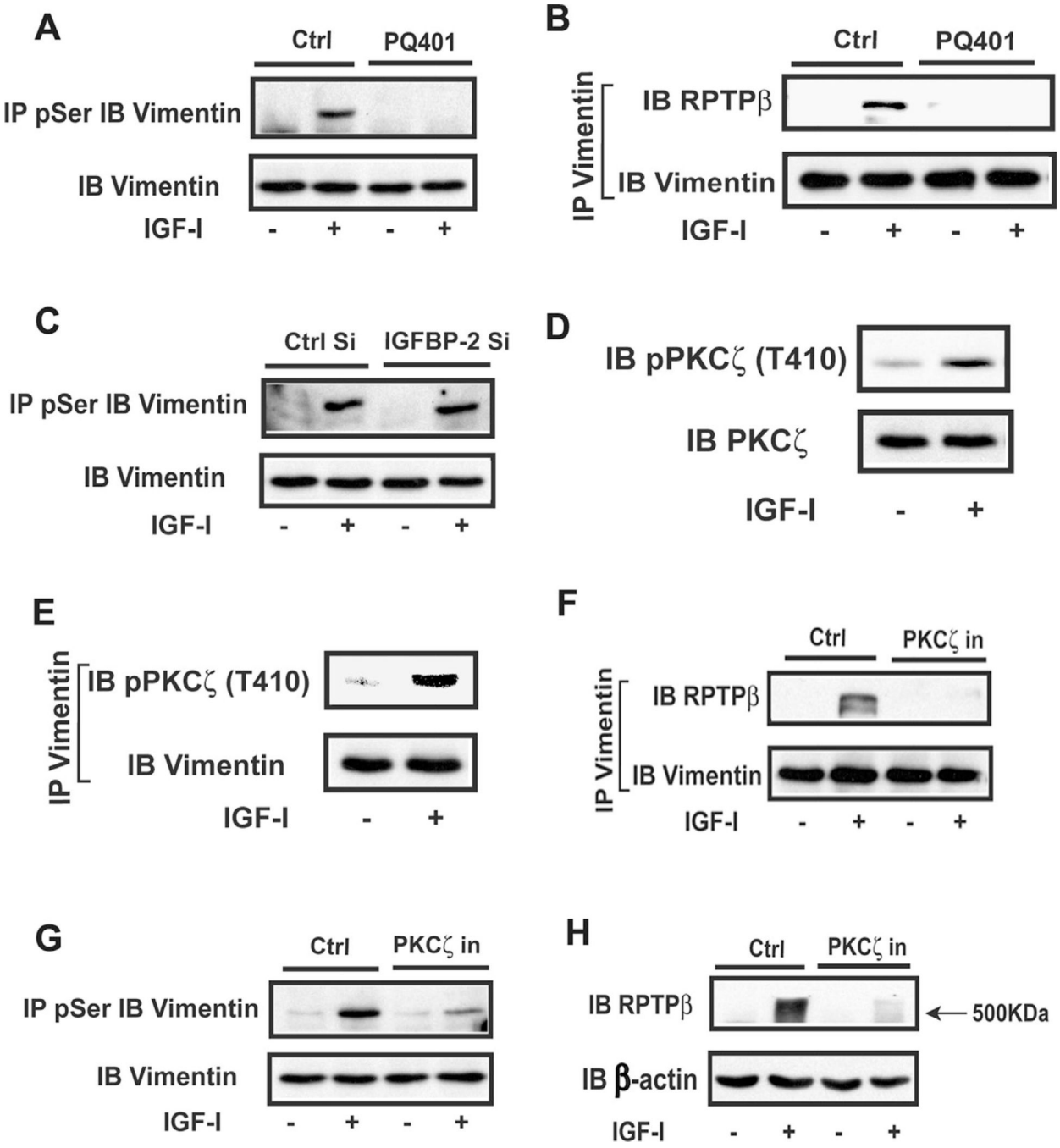

Fig. 2.

PKCζ phosphorylates vimentin which is required for IGF-I–stimulated vimentin/RPTPβ association and RPTPβ polymerization. (*A*, *B*) MC-3T3 cells were prepared as in the experiment shown in Fig. 1*A*. Cell lysates were immunoprecipitated with an anti-phosphoserine antibody (*A*) or anti-vimentin antibody (*B*) and immunoblotted with an anti-vimentin (*A*) or anti-RPTPβ antibody (*B*). (*C*) MC-3T3 cells expressing IGFBP-2 shRNA were prepared as in Fig. 1*A* then lysates were immunoprecipitated with an anti-phosphoserine antibody and immunoblotted with an anti-vimentin antibody. The same

amount of cell lysate was immunoblotted with an anti-vimentin antibody. (*D*, *E*) MC-3T3 cells were treated as in Fig. 1*A* then lysates were immunoblotted with an anti-pPKCζ (T410) or PKCζ antibody (*D*) or they were immunoprecipitated with an anti-vimentin antibody and immunoblotted with an anti-pPKCζ (T410) or vimentin antibody (*E*). (*F*–*H*) Cell lysates from MC-3T3 cells that had been exposed to differentiation medium for 3 days then incubated with a PKCζ inhibitor (PKCζ in) (10 μM) or PBS (Ctrl) for 2 hours with (+) or without (–) IGF-I for 5 min were immunoprecipitated with an anti-vimentin antibody (*F*) or an anti-phosphoserine antibody (*G*) and immunoblotted with an anti-RPTPβ antibody (*F*) or anti-vimentin antibody (*G*). Cell lysates were treated as described in Fig. 1*A* and immunoblotted with an anti-RPTPβ or anti-β-actin antibody (*H*).

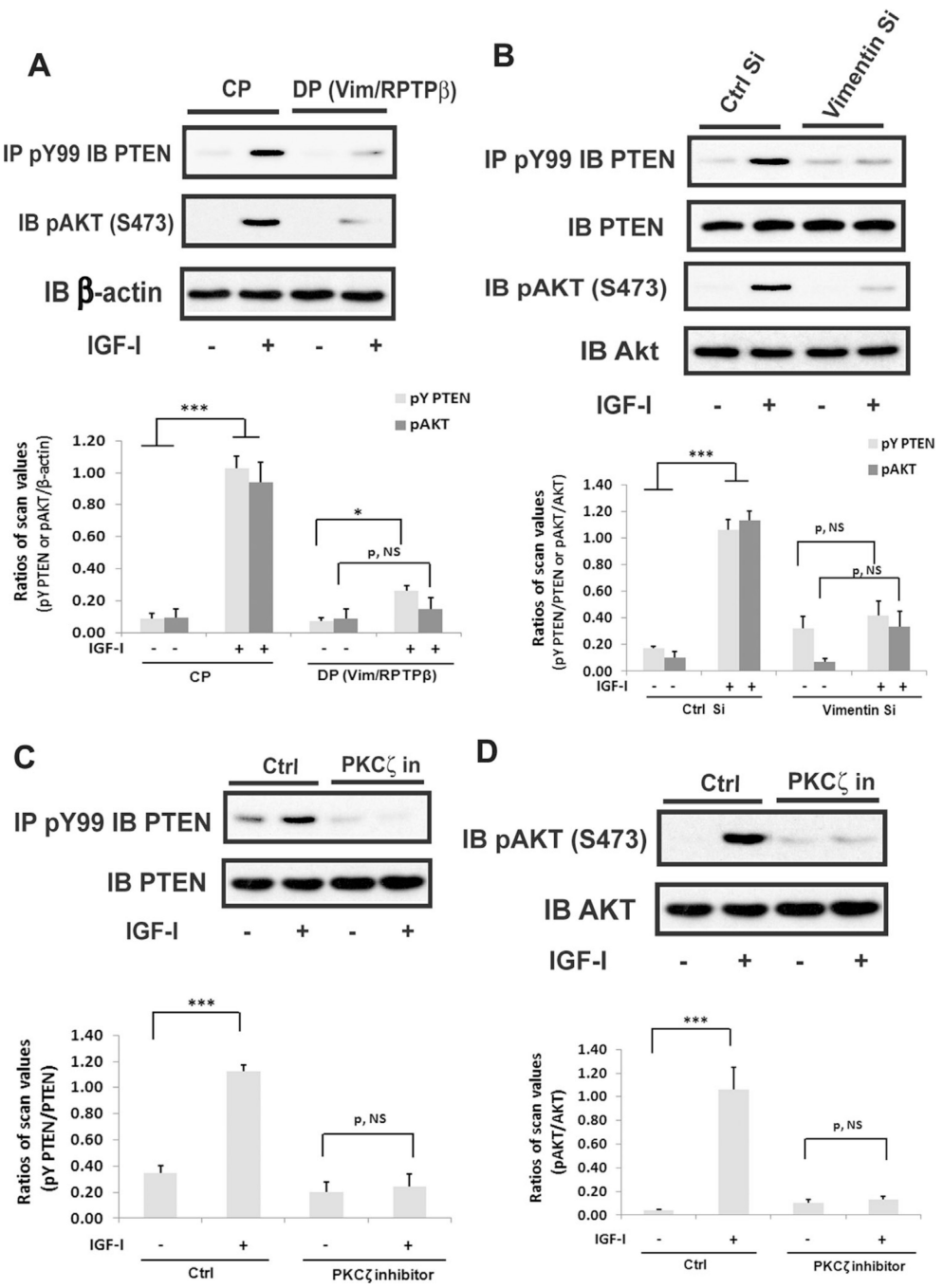

Fig. 3.

Disruption of vimentin/RPTPβ association impairs IGF-I-stimulated PTEN tyrosine phosphorylation and AKT activation. Quiescent MC-3T3 cells were exposed to differentiation medium for 3 days then incubated with (*A*) a CP or a vimentin/RPTPβ DP (10 μg/mL) for 2 hours (*B*) treated with a control siRNA (Ctrl Si) or vimentin siRNA (Vimentin Si) (30 pM) (*C, D*) incubated with a PKCζ inhibitor (PKCζ in) (10 μM) or PBS (Ctrl) for 2 hours) with (+) or without (-) IGF-I for 5 min. Cell lysates were immunoprecipitated with an anti-pY99 antibody and immunoblotted with an anti-PTEN

antibody (*A–C*) or were immunoblotted with an anti-pAKT (S473) antibody or anti-β-actin antibody or anti-AKT antibody (*A, B, D*). Each bar is the ratio of the scan value of the pY PTEN or pAKT band divided by the β-actin band (*A*), or the pY PTEN band divided by the PTEN band (*B, C*), or the pAKT band divided by the AKT band (*B, D*). ***$p < 0.001$ and *$p < 0.05$ indicate significant differences between two treatments. NS = no significant difference; CP = control peptide; DP = disrupting peptide.

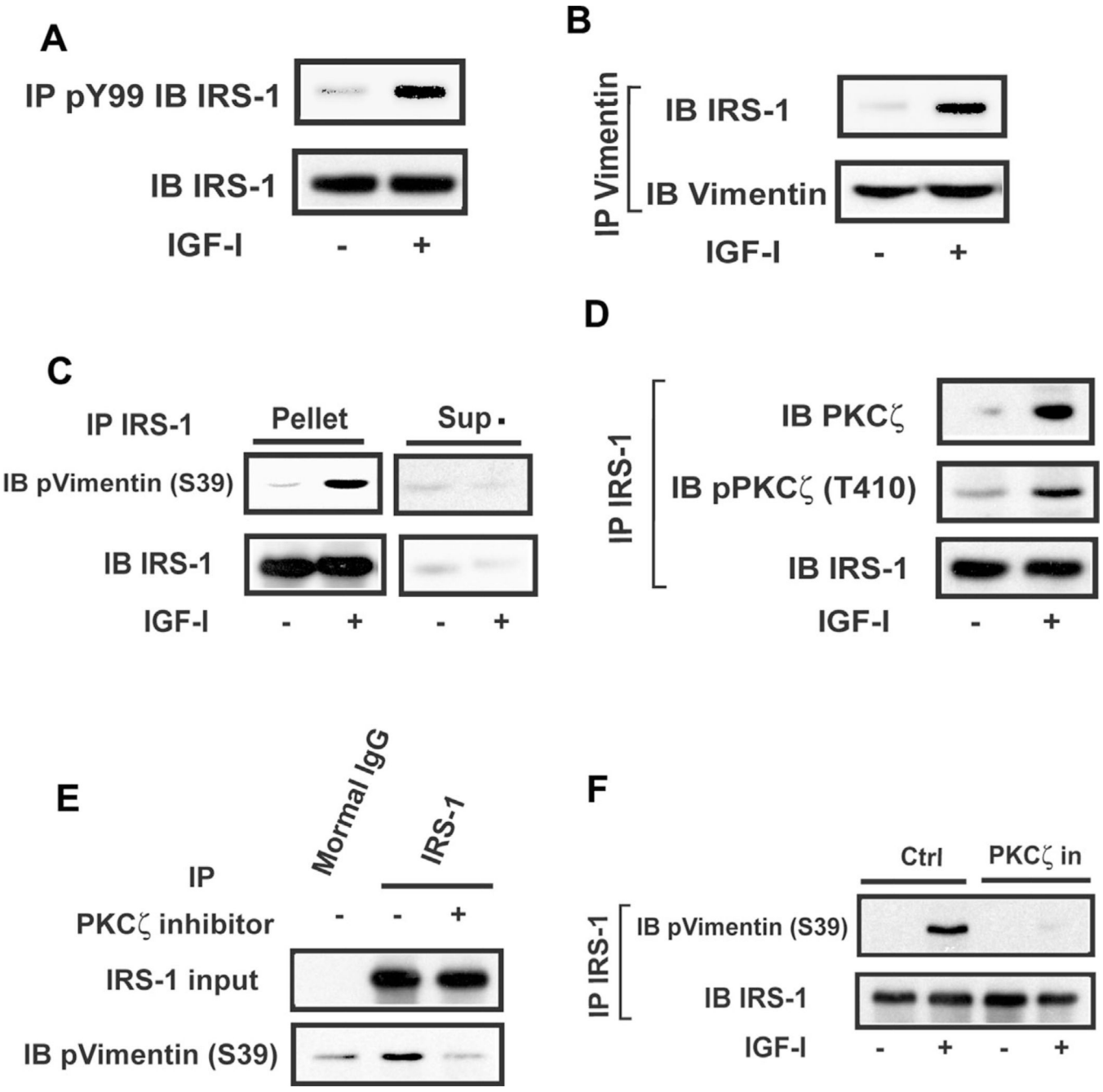

Fig. 4.

IRS-1 functions as a scaffold wherein PKCζ phosphorylates vimentin. (*A*–*D*) MC-3T3 cells were prepared as described in Fig. 1*A*. (*A*) Cell lysates were immunoprecipitated with anti-pY99 and immunoblotted with anti-IRS-1 or (*B*) immunoprecipitated with an anti-vimentin and immunoblotted with an anti-IRS-1. (*C*) Cell lysates were immunoprecipitated with an anti-IRS-1 antibody then both the immune complexes (pellet) and supernatants (Sup.) were immunoblotted with an anti-vimentin (phosphoS39) or IRS-1 antibody. (*D*) Cell lysates were immunoprecipitated with an anti-IRS-1 and immunoblotted with an anti-PKCζ, pPKCζ (T410), or IRS-1 antibody. (*E*) In vitro kinase assays were performed following a

procedure described in Materials and Methods. Cell lysate mixtures were immunoblotted with an anti-IRS-1 or vimentin (pS39) antibody. (*F*) MC-3T3 cells were exposed to differentiation medium for 3 days then incubated with a PKCζ inhibitor (PKCζ in) (10 μM) or PBS (Ctrl) for 2 hours with (+) or without (–) IGF-I for 5 min. Cell lysates were immunoprecipitated with an anti-IRS-1 and immunoblotted with an anti-Vimentin (pS39) or IRS-1 antibody.

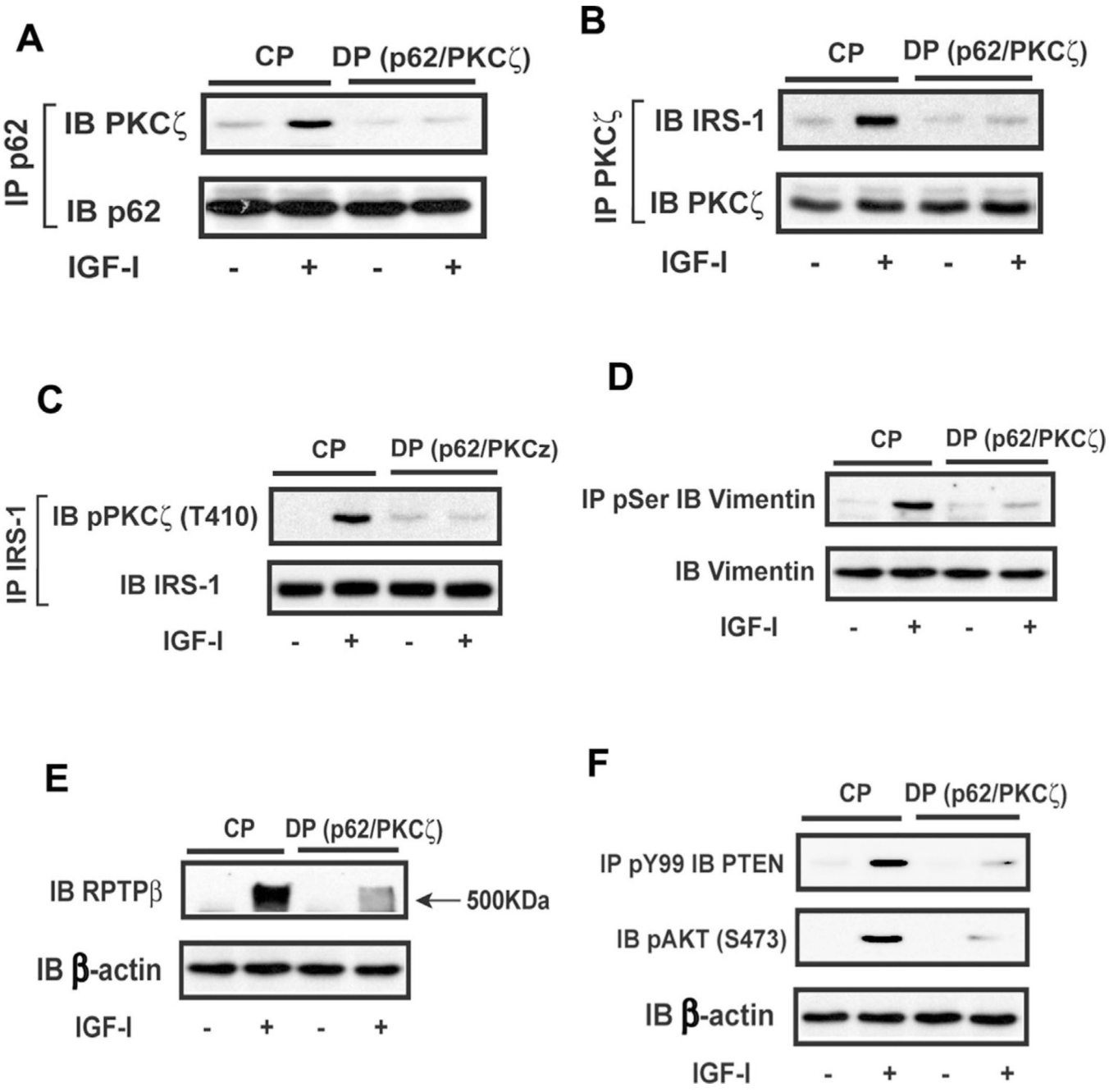

Fig. 5.

The recruitment of PKCζ to p62 is required for recruitment of PKCζ to IRS-1 and stimulation of downstream signaling. MC-3T3 cells were exposed to differentiation medium for 3 days and incubated with a CP or a p62/PKCζ DP (10 μg/mL) for 2 hours with (+) or without (–) IGF-I (100 ng/mL) for 5 min. (*A*) Cell lysates were immunoprecipitated with anti-p62 and immunoblotted with an anti-PKCζ or p62 antibody. (*B*) Cell lysates were immunoprecipitated with anti-PKCζ and immunoblotted with anti-IRS-1 or PKCζ. (*C*) Cell lysates were immunoprecipitated with anti-IRS-1 and immunoblotted with anti-pPKCζ (T410) or IRS-1. (*D*) Cell lysates were immunoprecipitated with anti-phosphoserine and

immunoblotted with anti-vimentin or immunoblotted with anti-vimentin as a loading control. (*E*) Cell lysates were prepared as described in Fig. 1*A* and were immunoblotted with anti-RPTPβ or anti-β-actin as a loading control. (*F*) Cell lysates were immunoprecipitated anti-pY99 and immunoblotted with anti-PTEN. The same amount of lysate was immunoblotted with anti-pAKT (S473) or β-actin. CP = control peptide; DP = disrupting peptide.

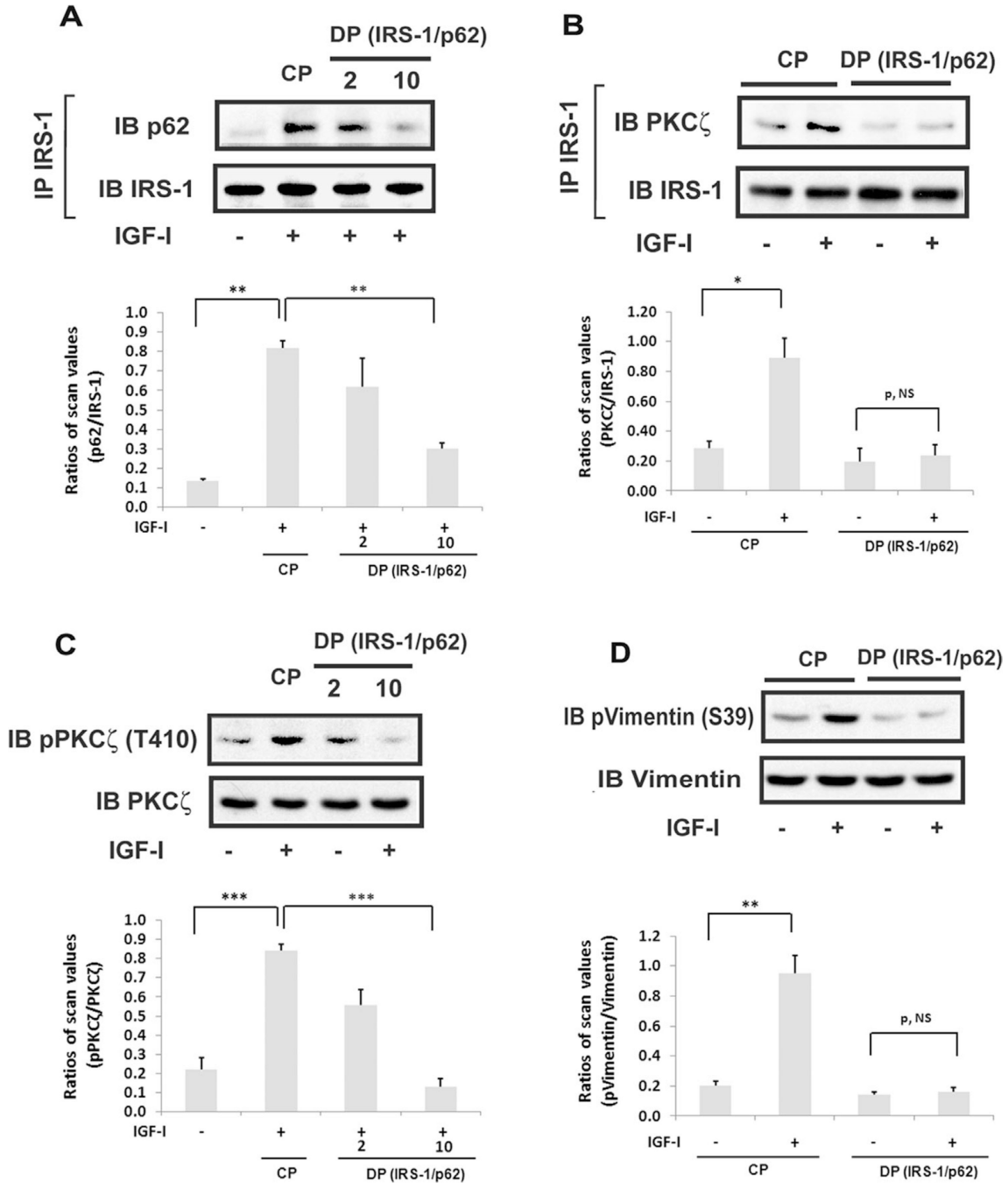

Fig. 6.

P62 binds to phosphotyrosines on IRS-1, which mediates PKCζ co-recruitment followed by vimentin phosphorylation.MC-3T3 cells were exposed to differentiation medium for 3 days and incubated with a CP or p62/IRS-1 DP (as indicated or 10 μg/mL) for 2 hours with (+) or without (-) IGF-I (100 ng/mL) for 5 min. (*A, B*) Cell lysates were immunoprecipitated with anti- IRS-1 then immunoblotted with anti-p62 (*A*), PKCζ (*B*), or IRS-1.(*C*) The same amount of lysate was immunoblotted with anti-pPKCζ (T410) or PKCζ antibody, (*D*) or anti-vimentin (pS39). Anti-vimentin was the loading control. Each bar is the ratio of the

scan value of the IRS-1 associated p62 (*A*) or PKCζ (*B*) band divided by IRS-1 band, or the pPKCζ (T410) band divided by the PKCζ band (*C*),or the pvimentin (S39) band divided by the vimentin band (*D*). ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ indicate significant differences between two treatments. NS = no significant difference; CP = control peptide; DP = disrupting peptide.

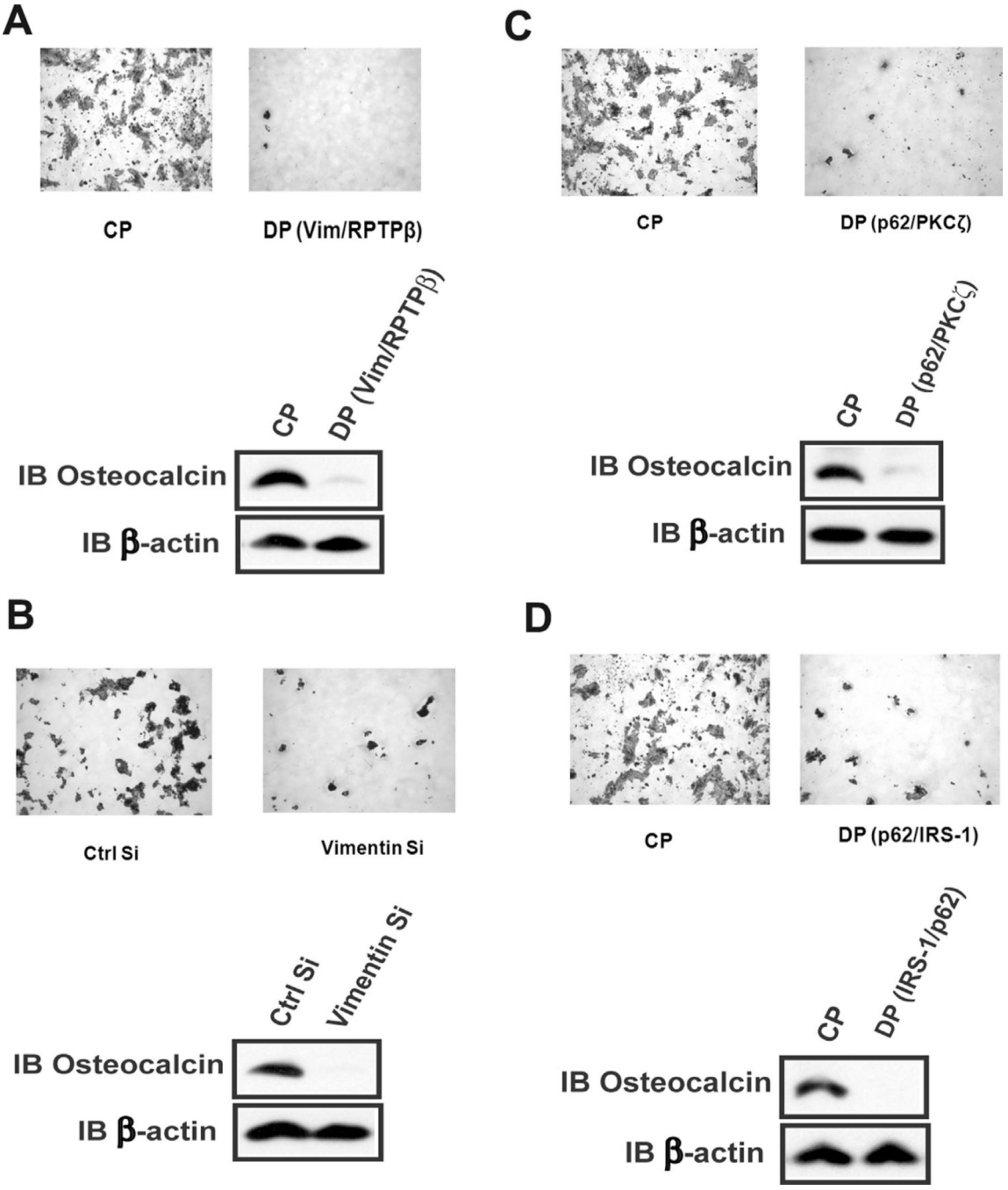

Fig. 7.

Disruption of the p62/PKCζ/Vimentin/RPTPβ signaling cascade impairs osteoblast differentiation. MC-3T3 cells were stained with Alizarin Red after a day 21 exposure to differentiation medium with (*A*) a CP or with a vimentin/RPTPβ DP (10 μg/mL) or (*B*) a control siRNA (Ctrl Si) or vimentin siRNA (vimentin Si) (30 pM) (*C*) or a p62/PKCζ DP (*D*) or IRS-1/p62 DPs. Cell lysates from the same treatments were immunoblotted with anti-osteocalcin or β-actin. CP = control peptide; DP = disrupting peptide.

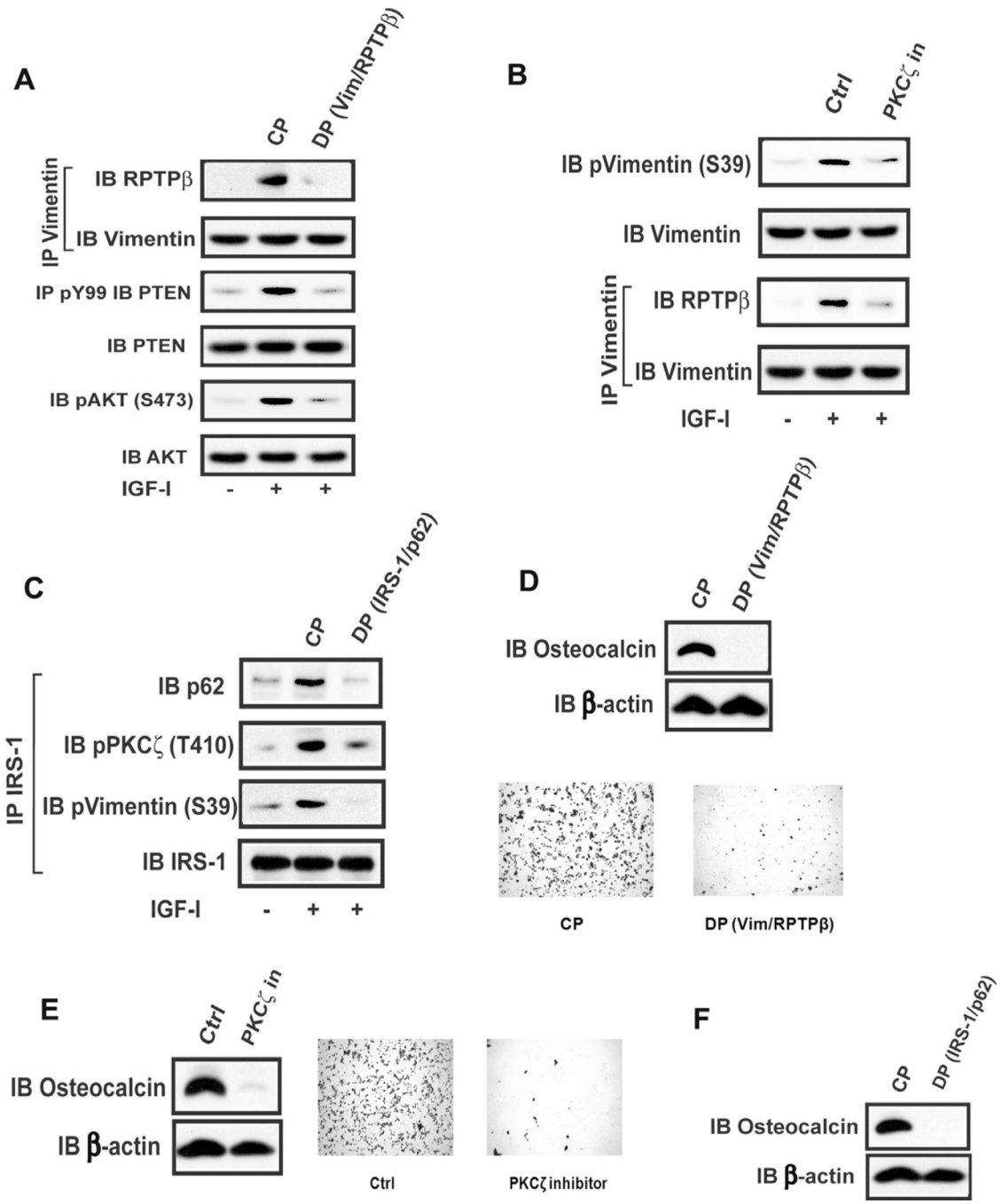

Fig. 8.

Activation of the p62/PKCζ/vimentin/RPTPβ signaling cascade is required for calvarial osteoblast differentiation. Calvarial osteoblasts isolated from wild-type mice were exposed to differentiation medium for 3 days (*A*–*C*) or 21 days (*D*–*F*). Cell lysates were prepared from quiescent cells treated with a CP or a vimentin/RPTPβ DP (10 μg/mL) (*A*, *D*) or PBS (Ctrl) or a PKCζ inhibitor (PKCζ in) (10 μM) (*B*, *E*) or IRS-1/p62 DPs (10 μg/mL) (*C*, *F*) with (+) or without (–) IGF-I for 5 min. (*A*) Cell lysates were immunoprecipitated with an anti-vimentin or pY99 antibody and immunoblotted with an anti-RPTPβ or vimentin or

PTEN antibody. The same amounts of lysates were immunoblotted with an anti-PTEN or pAKT (S473) or AKT antibody. (*B*) Cell lysates were immunoprecipitated with an anti-vimentin and immunoblotted with an anti-RPTPβ or vimentin antibody. The same amounts of lysates were immunoblotted with an anti-pvimentin (S39) or vimentin antibody. (*C*) Cell lysates were immunoprecipitated with an anti-IRS-1 antibody and immunoblotted with an anti-p62 or pPKCζ or pvimentin (S39) or IRS-1 antibody. (*D*, *E*) Cells were stained by Alizarin Red on day 21 after exposed to differentiation medium. (*D*–*F*) Cell lysates were immunoblotted with anti-osteocalcin or β-actin antibody. CP = control peptide; DP = disrupting peptide.

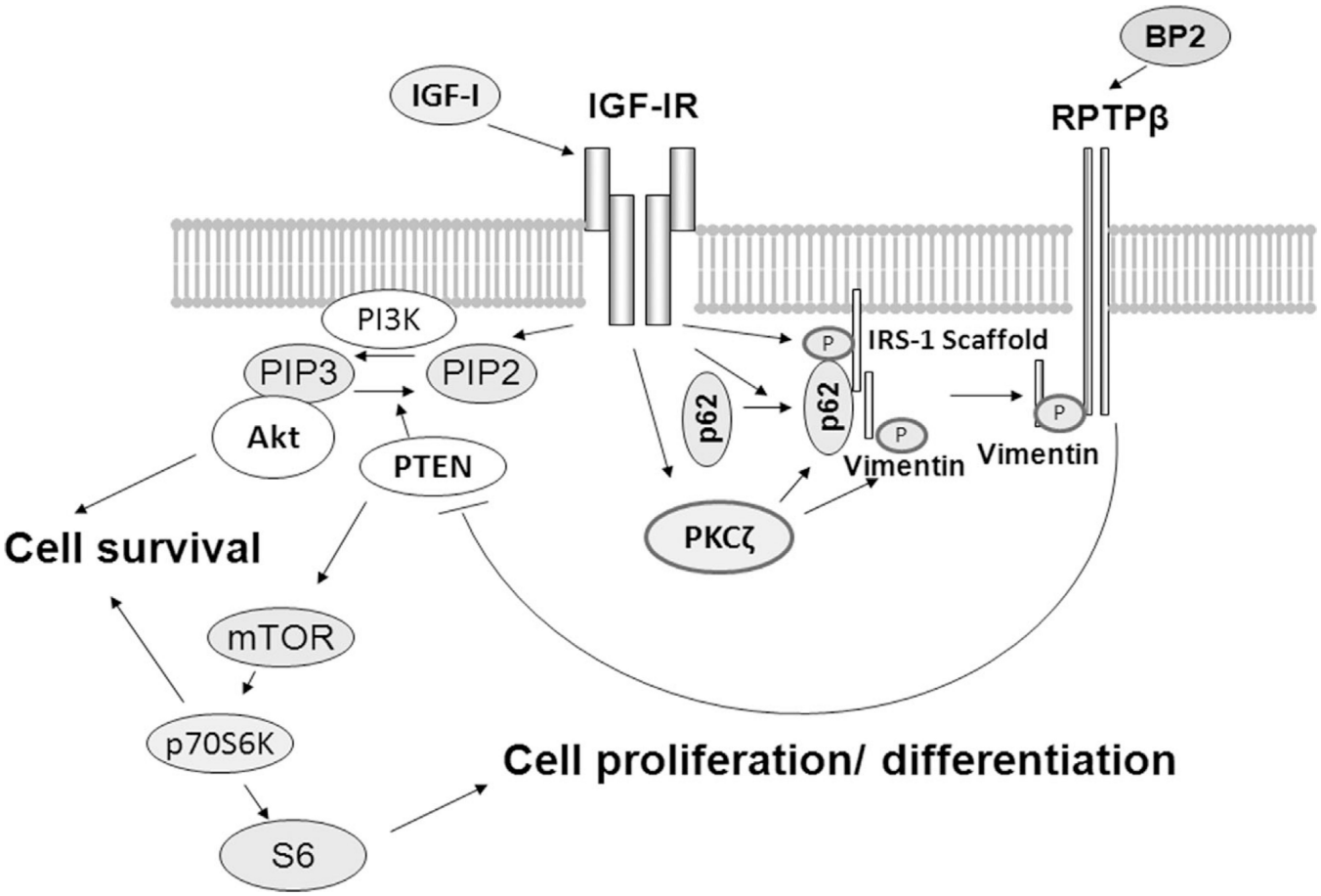

Fig. 9.

IGF-I and IGFBP-2 function cooperatively to stimulate vimentin/RPTPβ association, which enhances osteoblast differentiation. After exposure to differentiation medium, IGF-I stimulates IRS-1 tyrosine phosphorylation and PKCζ activation and p62/PKCζ association in osteoblasts. Phosphorylated tyrosines on IRS-1 provide binding sites for the SH2 domain containing protein, p62, which co-recruits PKCζ. Activated PKCζ phosphorylates vimentin that is localized on IRS-1, leading to vimentin/RPTPβ association. This association also requires the presence of IGFBP-2, and results in polymerization and inactivation of RPTPβ, leading to increased tyrosine phosphorylation of PTEN and enhanced activation of AKT. Consequently, these signaling events are required for osteoblast differentiation.