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Src Kinases Mediate VEGFR2 Transactivation by the Osteostatin Domain of PTHrP to Modulate Osteoblastic Function

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ABSTRACT

Parathyroid hormone-related protein (PTHrP) stimulates osteoblastic function through its N- and C-terminal domains. Since the osteogenic action of the latter domain appears to depend at least in part on its interaction with the vascular endothelial growth factor (VEGF) system, we aimed to explore the putative mechanism underlying this interaction in osteoblasts. Using native conditions for protein extraction and immunoblotting, we found that both PTHrP (107–139) and the shorter PTHrP (107–111) peptide (known as osteostatin), at 100 nM, promoted the appearance of a VEGF receptor (VEGFR) 2 protein band of apparent Mr. wt. 230 kDa, which likely represents its activation by dimer formation, in mouse osteoblastic MC3T3-E1 cells. Moreover, osteostatin (100 nM) maximally increased VEGFR2 phosphorylation at Tyr-1059 within 5–10 min in both MC3T3-E1 and rat osteoblastic osteosarcoma UMR-106 cells. This phosphorylation elicited by osteostatin appears to be VEGF-independent, but prevented by the VEGFR2 activation inhibitor SU1498 and also by the Src kinase inhibitors SU6656 and PP1. Furthermore, osteostatin induced phosphorylation of Src, extracellular signal-regulated kinase (ERK) and Akt with a similar time course to that observed for VEGFR2 activation in these osteoblastic cells. This osteostatin-dependent induction of ERK and Akt activation was abrogated by SU6656. Up-regulation of VEGF and osteoprotegerin gene expression as well as the pro-survival effect induced by osteostatin treatment were all prevented by both SU1498 and SU6656 in these osteoblastic cells. Collectively, these findings demonstrate that the osteostatin domain of C-terminal PTHrP phosphorylates VEGFR2 through Src activation, which represents a mechanism for modulating osteoblastic function. J. Cell. Biochem. 114: 1404–1413, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: OSTEOSTATIN; SRC KINASES; VASCULAR ENDOTHELIAL GROWTH FACTOR; OSTEOBLASTS

Parathyroid hormone (PTH)-related protein (PTHrP) is an important bone cytokine acting as a modulator of bone formation and remodeling [Datta and Abou-Samra, 2009]. PTHrP actions in bone are a consequence of the interaction of its N-terminal domain with the PTH/PTHrP receptor 1 (PPR); although domains other than this PTH-like domain might also contribute to the osteotropic effects of this protein [Toribio et al., 2010]. In this regard, in vitro studies demonstrate that the pentapeptide domain

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107–111 (known as osteostatin), comprising the N-terminus of the native C-terminal PTHrP (107–139) fragment, can directly affect osteoblastic function, apparently through interaction with a PPR-unrelated receptor [Valín et al., 2001; Lozano et al., 2010]. The molecular nature of this putative receptor is unknown, but it appears to signal through intracellular calcium/protein kinase C and extracellular signal-regulated kinases [Valín et al., 2001; Lozano et al., 2012b]. In addition, the C-terminal region of PTHrP might modulate its intracrine action(s) through the nuclear localization signal [de Miguel et al., 2001; Miao et al., 2008]. In any event, several recent studies have disclosed the osteogenic capacity of the osteostatin-containing domain of PTHrP in vivo in rodents. Hence, intermittent administration of a pharmacologic dose of the native peptide PTHrP (107–139) for at least two weeks osteoporotic mice prevented long-bone loss [de Castro et al., 2010, 2012; Lozano et al., 2011]. Moreover, this type of PTHrP (107–139) administration improved bone regeneration after marrow ablation in mice with glucocorticoid-induced or diabetic osteopenia [de Castro et al., 2010; Lozano et al., 2011]. Furthermore, very recently, we showed that osteostatin conferred osteoinductive properties to a ceramic implant in a bone fracture model in normal and osteoporotic rabbits [Trejo et al., 2010; Lozano et al., 2012a].

Vascular endothelial growth factor (VEGF) has proven to act as an important modulator of bone formation and regeneration [Schipani et al., 2009; Portal-Núñez et al., 2012]. In this regard, the stimulatory effects of PTHrP (107–139) and osteostatin on the latter process were found to occur associated with up-regulation of the VEGF system and increased neovascularization [de Castro et al., 2010; Trejo et al., 2010; Lozano et al., 2011, 2012a]. Both PTHrP (107–139) and osteostatin have been shown to rapidly increase VEGF gene expression in various osteoblastic cell types [Esbrit et al., 2000; Lozano et al., 2012b]. Also in this respect, the pro-survival and at least some differentiation-inducing actions of PTHrP (107–139) in human osteoblastic cells in vitro were abolished by a VEGF receptor (VEGFR2) tyrosine kinase inhibitor [de Gortázar et al., 2006; Alonso et al., 2008].

A recent report has shown that rapid and VEGF-independent phosphorylation of VEGFR2 occurs in human osteoblastic osteosarcoma MG-63 cells upon incubation with PTHrP (107–139) [Alonso et al., 2008]. In endothelial cells, VEGFR2 transactivation can be elicited by various angiogenic factors through a mechanism independent of VEGF ligands and involving Src kinases [Tanimoto et al., 2002; Fujita et al., 2006; Petreaca et al., 2007]. The latter are non-receptor protein tyrosine kinases with important roles in cell growth, survival, adhesion, and motility, which are regulated by a variety of growth factors and cytokines [Thomas and Brugge, 1997; Roskoski, 2004]. Recent studies support the contention that Src activation by phosphorylation at Tyr-416 promotes osteoblastic growth and survival, and might also affect osteoblast maturation [Marzia et al., 2000; Katz et al., 2011; Lausson and Cressent, 2011].

In the present study, we show the critical role of Src kinase activity in triggering an activation cascade involving VEGFR2, extracellular signal-regulated kinases (ERKs) and phosphatidylinositol-3 kinase (PI3K)/Akt, induced by osteostatin to modulate osteoblastic function.

MATERIALS AND METHODS

MATERIALS

Human PTHrP (107–139) was synthesized by F. Roncal, Ph.D. (Proteomics Unit, Centro Nacional de Biotecnología, Madrid). Osteostatin was from Bachem (Bubendorf, Switzerland). VEGFR2 phosphorylation inhibitor SU1498, Src inhibitor SU6656, the rabbit polyclonal anti-α-tubulin antibody, and etoposide were obtained from Sigma–Aldrich (St. Louis, MO). Src inhibitor PP1 and the VEGF binding inhibitor CBO-P11 were from Calbiochem (San Diego, CA). Recombinant human $VEGF_{165}$ and the rabbit polyclonal anti-VEGF antibody were from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies against Akt (Src-8312) and VEGFR2 (Src-505) were from Santa Cruz Biotechnology (Santa Cruz, CA). The following rabbit polyclonal antibodies against: phospho (p)-Tyr-1059 in VEGFR2, p-Ser473-Akt, p42/p44 (ERK 1/2), p-(Thr202/Tyr204)-ERK 1/2 and p-Tyr-416-Src (Cell Signaling Technology, Beverly, MA), and Src (Abcam, Cambridge, UK) were also used. NativePAGE™ Novex® 4–16% bis–Tris gel system was supplied by Invitrogen-Life Technologies (Carlsbad, CA). ECL-Western blotting kit, hyperfilm ECL and horseradish peroxidase-conjugated anti-rabbit IgG were all from Amersham Pharmacia Biotech (Buckinghamshire, UK).

CELL CULTURES

Mouse osteoblastic MC3T3-E1 cells (CRL-2593; ATCC, Manassas, VA) and rat osteoblastic osteosarcoma UMR-106 cells (CRL-1661; ATCC) were grown in α-minimum essential medium and Dulbecco's Modified Eagle's Medium, respectively, supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin and 2 mM glutamine in 5% CO_2 at 37°C. For experiments, cells at 80% confluence were maintained overnight in serum-depleted medium. Thereafter, cells were incubated for different time periods with PTHrP (107–139), osteostatin (each at 100 nM), or $VEGF_{165}$ (6 ng/ml), in the presence or absence of SU1498, SU6656, or PP1 (each at 10 μM) [added at least 2 h before each agonist (or vehicle)]. A neutralizing VEGF antibody (0.5 μg/ml) or the VEGF binding inhibitor CBO-P11 (20 μM) [Zilberberg et al., 2003] was added to the cell incubation medium to reduce phosphorylated VEGFR2 signal (control) by endogenous VEGF. The concentrations of these VEGF inhibitors used were previously found to antagonize VEGF bioactivity in various osteoblastic cell preparations including MC3T3-E1 cells [Alonso et al., 2008; Lozano et al., 2012b]. Vehicle-treated cells (control) were consistently assessed at the time corresponding to maximal signal triggered by the agonist in each case.

In experiments to assess changes in cell viability, cells were seeded at a density of 2×10^4 cells/cm^2. After 24 h, cells were refed with fresh medium, and pre-incubated with SU6656 or SU1498 (each at 10 μM) for 2 h, followed by addition of osteostatin (at 100 nM) (or vehicle) for 6 h. Subsequently, cells were exposed to etoposide (50 μM), an anti-cancer agent which induces apoptosis [Bellido et al., 2003], or the corresponding vehicle, overnight in FBS-depleted medium. After cell stimulation, non-adherent cells were collected and pooled with adherent cells (after gentle trypsinization), and then mixed with 0.4% trypan blue solution (1:1, v/v). The

number of total cells and that of those exhibiting intracellular staining (nonviable cells) were counted in a hemocytometer, and the percentage of cell viability was then determined.

PROTEIN EXTRACTION AND IMMUNOBLOTTING

In order to assess whether osteostatin might promote VEGFR2 activation by inducing this receptor protein dimerization, MC3T3-E1 cells were exposed to PTHrP (107–139), osteostatin (each at 100 nM) or $VEGF_{165}$ (6 ng/ml) for 2–20 min. Thereafter, cell extracts were obtained by adding NativePAGE™ Sample Buffer (Invitrogen-Life Technologies) with 1% dodecylmaltoside followed by centrifugation at 100,000*g* for 30 min. To evaluate changes in phosphorylated proteins in non native conditions, osteoblastic cell extracts were obtained in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecylsulfate (SDS). In both native and non-native conditions, extraction buffer was supplemented with protease inhibitor cocktail Set III and phosphatase-inhibitor cocktail Set II (Calbiochem). Protein content was determined by Bradford's method (Thermo Fisher Scientific, Rockford, IL), using bovine serum albumin (BSA) as standard.

Protein extracts (50 μg) were separated on 4–16% bis–Tris gels under non-reducing conditions (NativePAGE™ system). To evaluate changes in protein phosphorylation, equal amounts of cell extracts were subjected to SDS–PAGE in reducing conditions on 6.5% polyacrilamide gels (non-native conditions). After electrophoresis, proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% BSA in 100 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Tween-20, and then incubated overnight at 4°C with the following primary antibodies (each at 1:1,000 dilution): VEGFR2 or p-Tyr1059-VEGFR2; Src or p-Tyr416-Src; ERK 1/2 or p-(Thr202/Tyr204)-ERK 1/2; and Akt or p-Ser473-Akt. As loading control, an anti-α-tubulin (at 1:5,000 dilution) or anti-β actin antibody (at 1:500 dilution) was used; the membranes were subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG, developed by ECL chemiluminiscence, and fluorogram bands were quantified by densitometry.

REAL-TIME PCR ANALYSIS

Cell total RNA was extracted with Trizol (Foster City, CA). Aliquots (0.5–4 μg) were then retrotranscribed with the cDNA High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using an ABI PRISM 7500 system (Applied Biosystems) and a previously described protocol [Lozano et al., 2010, 2011, 2012b]. Unlabeled mouse or rat specific primers for VEGF and osteoprotegerin (OPG), and TaqMan^{MGB} probes were obtained by Assay-by-Design^{SM} (Applied Biosystems). The mRNA copy numbers were calculated for each sample by using the cycle threshold (Ct) value, and normalized against 18S rRNA, a housekeeping gene which was amplified in parallel with the tested genes [Lozano et al., 2010, 2011, 2012b].

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM throughout the text. Differences between experimental and control conditions were analyzed by using unpaired two-tail *t*-test. $P < 0.05$ was considered significant.

RESULTS

OSTEOSTATIN TARGETS TYR-1059 TO INDUCE VEGFR2 TRANSACTIVATION THROUGH Src PHOSPHORYLATION IN OSTEOBLASTIC CELLS

It is well known that VEGFR2 activation by VEGF ligands induces the formation of homo- and heteromeric receptor complexes (dimerization), which in turn triggers kinase activation [Matsumoto and Claesson-Welsh, 2001]. Thus, in the present study, we first aimed to examine whether osteostatin might be able to induce this receptor dimerization in osteoblastic cells by using Western blotting with an anti-VEGFR2 antibody. We here show that treatment of MC3T3-E1 cells with $VEGF_{165}$, at 6 ng/ml, for only 2 min promoted the appearance of an immunoblotted protein band of apparent Mr. wt. 230 kDa after protein separation in native conditions, likely representing VEGFR2 dimer formation, which disappeared at 20 min (Fig. 1). This is consistent with the rapid and transient VEGFR2 phosphorylation by its ligand observed in other osteoblastic cell preparations and endothelial cells [Zhang et al., 2003; Alonso et al., 2008]. Moreover, a similar band was observed by analyzing protein extracts in the same way following treatment of MC3T3-E1 cells with the native PTHrP (107–139) fragment or osteostatin, each at 100 nM, for 10–20 min (Fig. 1). These data add credence to the hypothesis that osteostatin promotes VEGFR2 activation.

Using Western analysis for phosphorylation of VEGFR2 at Tyr-1059 in protein lysates separated by SDS–PAGE in non-native conditions, we also found that osteostatin, at 100 nM, was able to maximally induce phosphorylation of a 200 kDa VEGFR2 protein band at 5–10 min in both non-transformed osteoblastic MC3T3-E1 cells and osteoblastic osteosarcoma UMR-106 cells (Fig. 2A,C). This tyrosine residue corresponds to an autophosphorylation site following VEGFR2 activation [Dougher-Vermazen et al., 1994; Dougher and Terman, 1999; Zachary and Gliki, 2001; Zeng et al.,

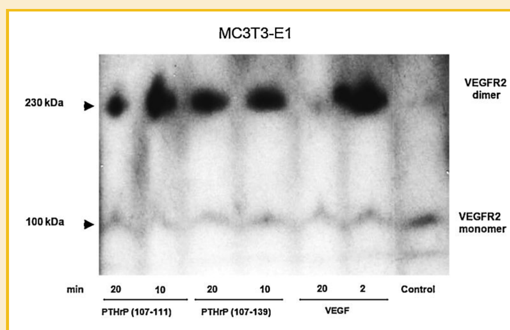

Fig. 1. Osteostatin, similarly to VEGF ligand, promoted VEGFR2 dimerization in MC3T3-E1 cells. These cells were exposed to PTHrP (107–139) or PTHrP (107–111) (osteostatin) (at 100 nM) for 10 and 20 min, or to $VEGF_{165}$ (6 ng/ml) for 2 and 20 min. Western analysis of protein extracts was then performed using an anti-VEGFR2 antibody after electrophoresis in native conditions, as described in Materials and Methods Section. Each agonist promoted the appearance of a protein band of apparent Mr. wt. 230 kDa, which likely corresponds to VEGFR2 dimer. An autoradiogram of a representative immunoblot is shown, corresponding to results obtained in at least three independent experiments.

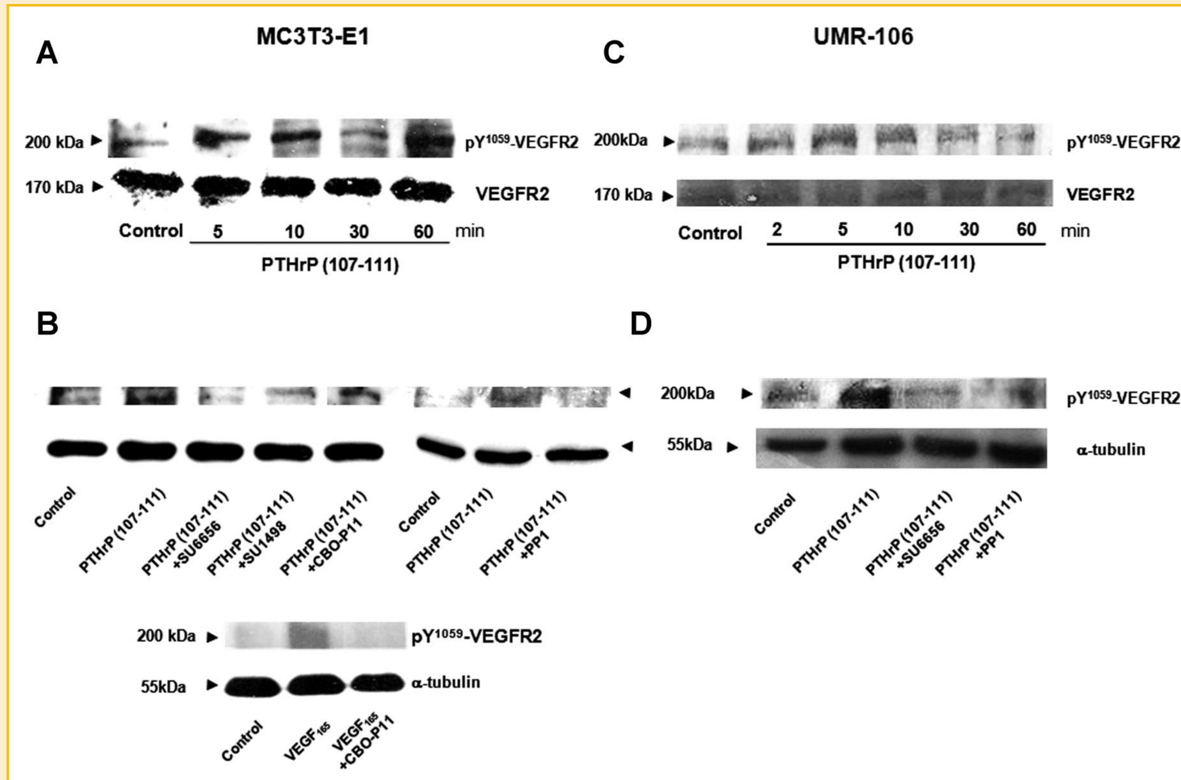

Fig. 2. PTHrP (107–111) (osteostatin) rapidly induced phosphorylation of VEGFR2 at Tyr-1059 in both MC3T3-E1 cells and UMR-106 cells. These cells were exposed to osteostatin (100 nM) for different time periods (A,C) or for 10 min (B,D). When present, the VEGFR2 kinase activity inhibitor SU1498 (10 μM) or the VEGF binding inhibitor CBO-P11 (20 μM) (B), or the Src kinase inhibitors SU6656 and PP1 (each at 10 μM) (B and D) were added at least 2 h before osteostatin. As a positive control, MC3T3-E1 cells were exposed to $VEGF_{165}$ (6 ng/ml) for 2 min in the absence or presence of CBO-P11 (B, bottom). A neutralizing VEGF antibody (0.5 μg/ml) was added to the incubation medium of MC3T3-E1 cells or UMR-106 cells to reduce the control signal. Representative autoradiograms are shown (VEGFR2 and p-VEGFR2 appeared at 170 and 200 kDa, respectively), corresponding to results obtained in at least three independent experiments in duplicate.

2001; Matsumoto and Mugishima, 2006]. Moreover, we confirmed that this phosphorylation triggered by osteostatin in these osteoblastic cells requires direct VEGFR2 transactivation since it occurred in the presence of either a neutralizing VEGF antibody or the VEGF binding inhibitor CBO-P11, and it was abrogated by SU1498, a highly selective inhibitor of VEGFR2 activation [Tanimoto et al., 2002; Zhang et al., 2003] (Fig. 2). Interestingly, this osteostatin-induced phosphorylation was found to be inhibited by the Src inhibitors, SU6656 and PP1, in both osteoblastic cell types (Fig. 2B,D). Neither of these inhibitors alone had any significant effect on this phosphorylation (not shown). Moreover, consistent with these findings, osteostatin was found to induce Src activation with a time course comparable to that seen for VEGFR2 phosphorylation in both osteoblastic cell lines studied (Fig. 3). These data indicate that Src can be targeted by osteostatin to induce VEGFR2 transactivation.

BOTH ERK AND AKT ACTIVATION OCCURS DOWNSTREAM OF Src-DEPENDENT VEGFR2 PHOSPHORYLATION BY OSTEOSTATIN IN OSTEOBLASTIC CELLS

The important role of ERK and PI3K/Akt signaling following VEGFR2 activation is well known [Gerber et al., 1998; Dayanir et al., 2001; Matsumoto and Claesson-Welsh, 2001; Tanimoto et al., 2002; Fujita et al., 2006]. Here, we further explored the relationship between VEGFR2 phosphorylation and ERK and Akt activation by osteostatin in osteoblastic cells. We found that phosphorylation of both kinases was promoted by osteostatin within a similar time frame as that observed for VEGFR2 and Src activation in MC3T3-E1 cells (Fig. 4). Moreover, increased ERK 1/2 and Akt phosphorylation by osteostatin was abrogated by SU6656 in both osteoblastic cell lines studied (Fig. 5). Consistent with previous findings in endothelial cells [Kroll and Waltenberger, 1997; Gerber et al., 1998; Pedram et al., 1998], SU6656 failed to affect Akt phosphorylation but abrogated ERK 1/2 phosphorylation elicited by $VEGF_{165}$ in MC3T3-E1 cells (Fig. 5A,B).

Src-DEPENDENT VEGFR2 PHOSPHORYLATION MEDIATES VARIOUS EFFECTS OF OSTEOSTATIN RELATED TO OSTEOBLASTIC FUNCTION

We next evaluated the putative role of Src-mediated VEGFR2 transactivation by osteostatin in modulating genes that are important for osteoblastic function. We found that this peptide up-regulated the gene expression of VEGF and OPG, an important modulator of bone turnover [Boyce and Xing, 2008], at 3–6 h in MC3T3-E1 cells (Fig. 6). Similarly, osteostatin was shown to induce

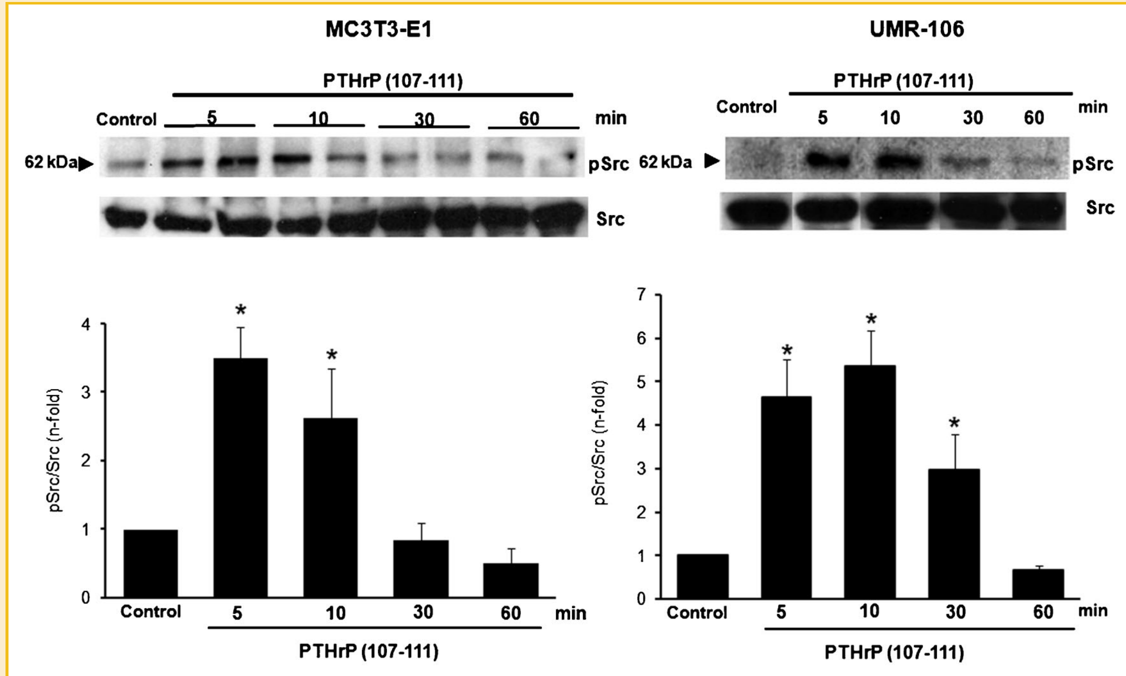

Fig. 3. PTHrP (107–111) (osteostatin) elicited Src phosphorylation in both MC3T3-E1 and UMR-106 cells. Cells were treated with osteostatin (100 nM) for different time periods, and then protein extracts were analyzed by Western blot. A neutralizing VEGF antibody (0.5 μg/ml) was added to the cell incubation medium to reduce putative Src phosphorylation by endogenous VEGF in control condition. Representative autoradiograms are shown. Corresponding densitometric data represent mean ± SEM of at least three independent experiments in duplicate. $^{*}P < 0.05$ versus control.

OPG mRNA expression at 3 h in UMR-106 cells (Fig. 7). In addition, we found that this up-regulation was inhibited by both SU1498 and SU6656 in these osteoblastic cells (Fig. 7). We also examined whether osteostatin would also affect osteoblast survival through Src-dependent VEGFR2 activation. Pre-incubation with osteostatin was found to inhibit the cell death induced by etoposide in both osteoblastic cell lines; and this pro-survival action was prevented by both SU1498 and SU6656 (Fig. 8).

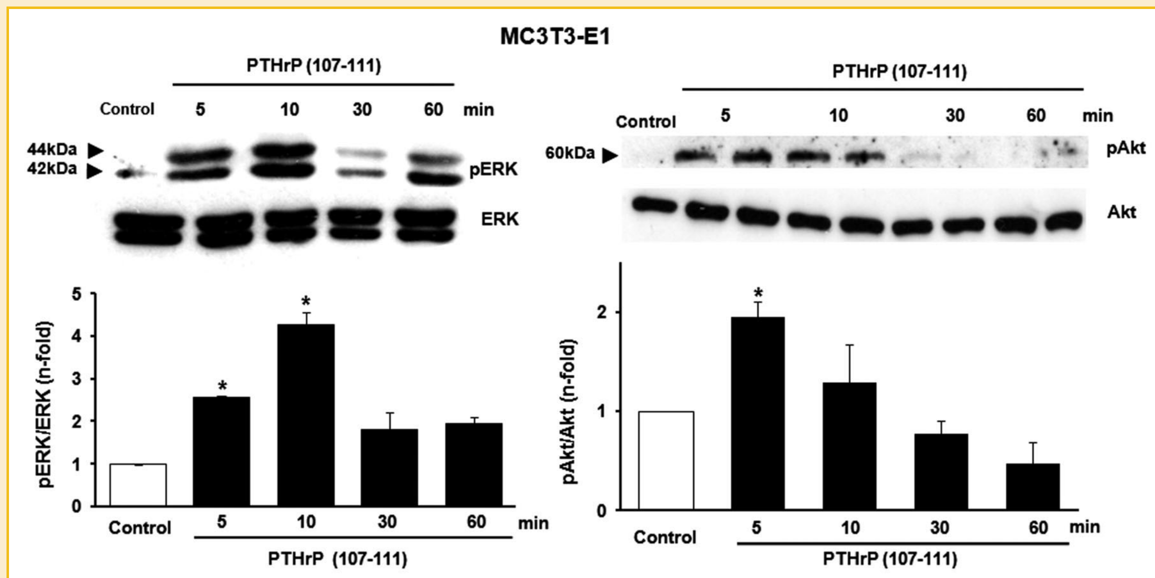

Fig. 4. Phosphorylation of ERK and Akt was induced by PTHrP (107–111) (osteostatin) in MC3T3-E1 cells. These cells were incubated with osteostatin (100 nM) for different time periods, and then protein extracts were analyzed by Western blot. A neutralizing VEGF antibody (0.5 μg/ml) was added to the cell incubation medium to reduce putative phosphorylation signal triggered by endogenous VEGF in each control condition. Representative autoradiograms are shown, and corresponding densitometric data represent mean ± SEM of three independent experiments in duplicate. $^{*}P < 0.05$ versus control.

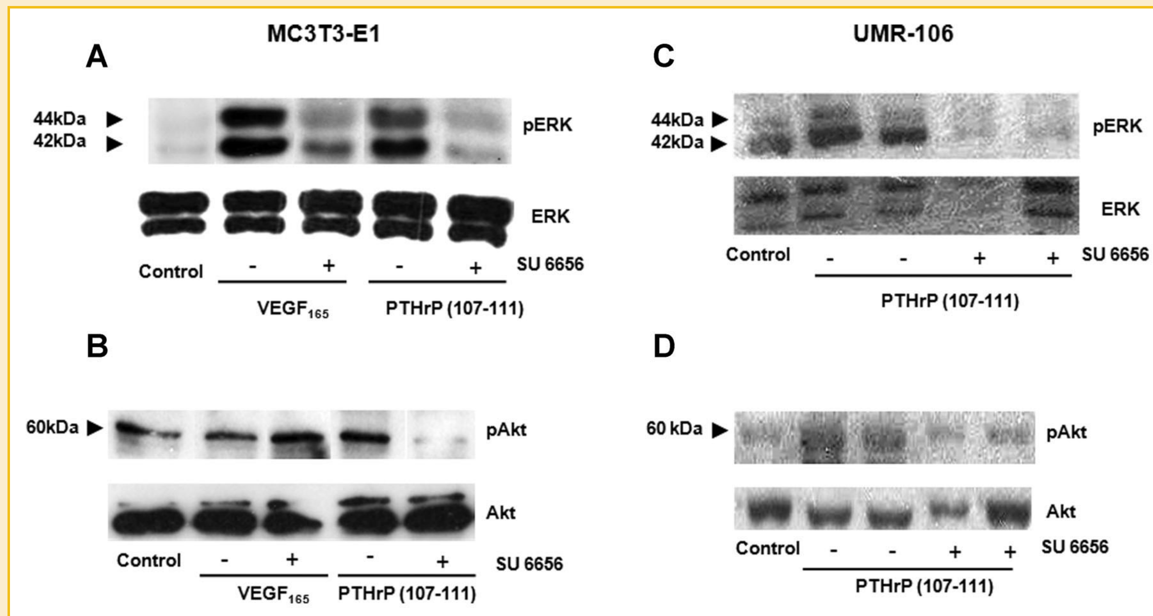

Fig. 5. ERK and Akt phosphorylation triggered by PTHrP (107–111) (osteostatin) were inhibited by a Src kinase inhibitor in both MC3T3-E1 and UMR-106 cells. Cells were treated with osteostatin (100 nM) for 10 min or $VEGF_{165}$ (as a positive control) (6 ng/ml) for 2 min (A,B), with or without the Src kinase inhibitor SU6656 (10 μM) (added 2 h prior to osteostatin), and protein extracts were then analyzed by Western blot. A neutralizing VEGF antibody (0.5 μg/ml) or the VEGF binding inhibitor CBO-P11 (20 μM) was added to the incubation medium of MC3T3-E1 cells or UMR-106 cells, respectively, to prevent putative phosphorylation by endogenous VEGF in each control condition. Representative autoradiograms shown correspond to results obtained in at least three independent experiments in duplicate.

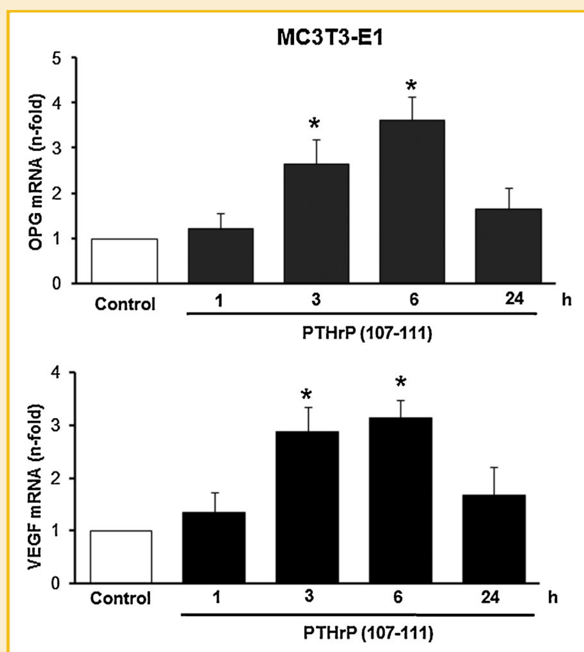

Fig. 6. Gene expressions of OPG and VEGF was up-regulated by PTHrP (107–111) (osteostatin) in MC3T3-E1 cells. These cells were treated with osteostatin (100 nM) for different time periods, and subsequently mRNA levels were assessed by real-time PCR. Results are mean ± SEM of three independent experiments in triplicate. $^{*}P < 0.05$ versus control.

DISCUSSION

In the present study, we report the role of Src in VEGFR2 transactivation by osteostatin as a mechanism to modulate osteoblastic function. Osteostatin was found to rapidly induce both Src and VEGFR2 phosphorylation in two osteoblastic cell lines. Recently, it was reported that the osteostatin-containing PTHrP (107–139) fragment activates VEGFR2 by a mechanism apparently independent of VEGF ligands in human osteoblastic cells [Alonso et al., 2008]. Our data herein, using osteostatin as agonist, further support this notion.

A variety of factors can transactivate VEGFR2 by a VEGF-independent mechanism including Src activation in endothelial cells [Chou et al., 2002; Tanimoto et al., 2002; Jin et al., 2003; Zhang et al., 2003; Fujita et al., 2006; Petreaca et al., 2007]. Src kinases play important roles in a variety of cell functions [Roskoski, 2004], and interact with a variety of growth factors and integrin receptors [Thomas and Brugge, 1997]. These kinases can directly phosphorylate epidermal growth factor receptor [Biscardi et al., 1999; Bromann et al., 2004], and this transactivation regulates important cellular events such as epithelial–mesenchymal transition and survival [Ardura et al., 2010; Hobbs et al., 2011]. Several studies also indicate that Src activation might be a mechanism to modulate osteoblast growth and function [Marzia et al., 2000; Katz et al., 2011; Lausson and Cressent, 2011; Chen et al., 2012a].

We show here that osteostatin induced phosphorylation of VEGFR2 at Tyr-1059, an autophosphorylated residue in its kinase domain [Dougher and Terman, 1999; Takahashi et al., 2001; Zachary and Gliki, 2001; Zeng et al., 2001; Matsumoto and Mugishima,

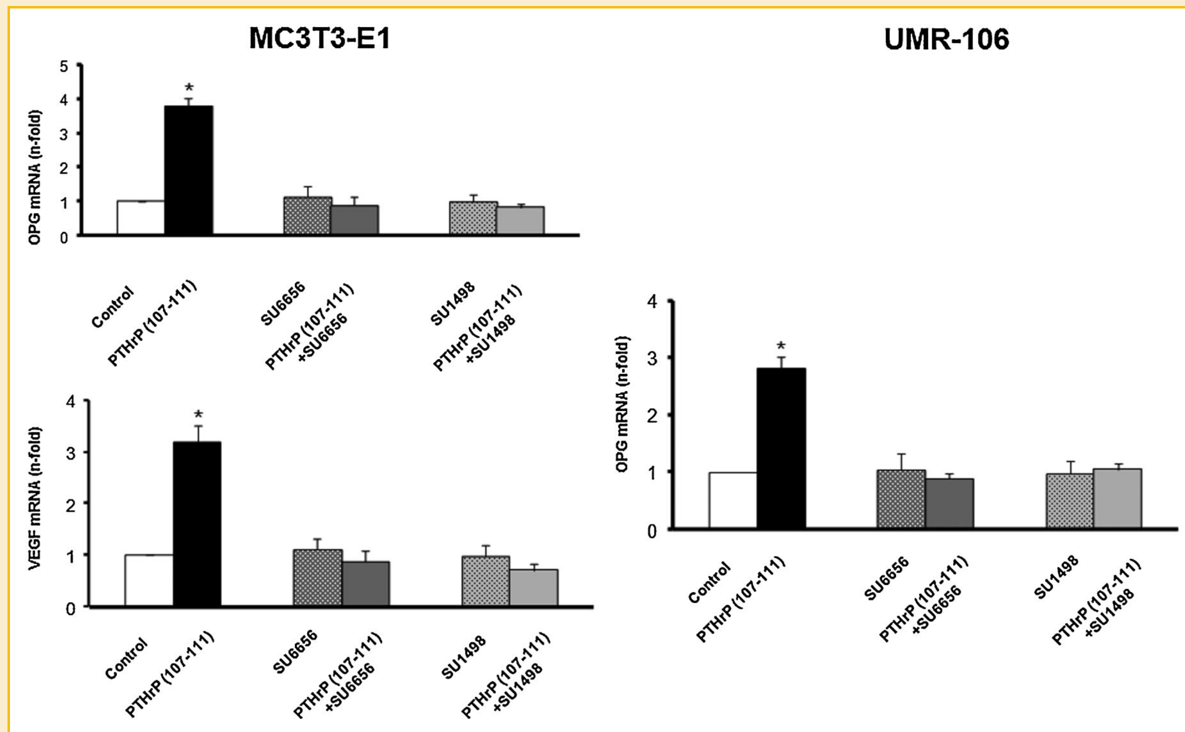

Fig. 7. Up-regulation of OPG and VEGF by PTHrP (107–111) (osteostatin) were inhibited by both Src kinase and VEGFR2 kinase activity inhibitors in osteoblastic cells. MC3T3-E1 and UMR-106 cells were exposed (or not, control) to osteostatin (100 nM) for 3–6 h and, subsequently, mRNA levels were assessed by real-time PCR. When present, SU1498 or SU6656 (each at 10 μM) were added 2 h before osteostatin. Values are mean ± SEM of at least three independent experiments in triplicate. $^{*}P < 0.05$ versus all the other conditions.

2006; Koch and Claesson-Welsh, 2012]. This tyrosine is essential for the receptor tyrosine kinase activity of VEGFR2 since its replacement by a phenylalanine residue leads to activity loss [Dougher and Terman, 1999; Kendall et al., 1999]. Hence, Tyr-1059 is unlikely to have been directly phosphorylated by Src. Instead, osteostatin activation of the latter kinase may have promoted phosphorylation of non-autophosphorylated tyrosine residues leading to VEGFR2 activation through Tyr-1059 autophosphoryla-

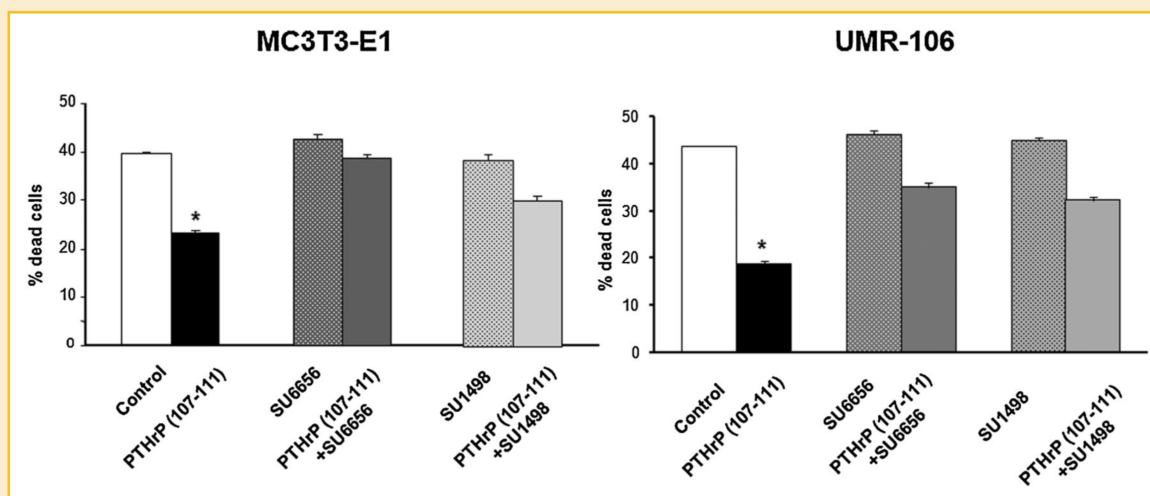

Fig. 8. The pro-survival effect of PTHrP (107–111) (osteostatin) was inhibited by Src kinase and VEGFR2 kinase activity inhibitors in osteoblastic cells. MC3T3-E1 and UMR-106 cells were exposed to osteostatin (100 nM) for 6 h, with or without SU1498 or SU6656 inhibitors (each at 10 μM), added 2 h prior to the peptide; subsequently, etoposide (50 μM) was added overnight before assessing cell viability. White bars correspond to cells treated with etoposide alone. Cell death in etoposide-untreated controls was <10% in both cell types (not shown). Results are mean ± SEM of at least three independent experiments in triplicate. $^{*}P < 0.05$ versus all the other conditions.

tion. In addition, Src activation could also occur downstream of osteostatin-induced VEGFR2 transactivation in osteoblastic cells, as reported for VEGF and other VEGF-independent agonists of this receptor in endothelial cells [Chou et al., 2002; Tanimoto et al., 2002; Jin et al., 2003; Fujita et al., 2006; Petreaca et al., 2007]. Previous in vitro studies have shown that osteostatin can directly target a high affinity PPR-unrelated receptor to trigger Ca^{2+} uptake, apparently independent of Gs or Gi-mediated activation of phospholipase C, in UMR-106 cells [Valín et al., 2001]. It is presently unknown whether this putative receptor might also be responsible for the activation of Src by osteostatin as shown here in this cell line and another non-transformed osteoblastic cell line. Of note in this regard, the angiogenic lipid, sphingosine-1-phosphate, has been reported to independently transactivate VEGFR2 through Ca^{2+}- and Src-dependent pathways in endothelial cells [Tanimoto et al., 2002]. Further investigations are needed to clarify the possible cooperation between these pathways in the mechanisms whereby osteostatin transactivates VEGFR2 in osteoblastic cells. In any event, the present findings demonstrate that activation of ERK and Akt signaling pathways takes place downstream of Src activation by osteostatin in osteoblastic cells. The fact that osteostatin rapidly triggered VEGFR2 phosphorylation at Tyr-1059, a critical site for ERK and PI3K/Akt activation [Zeng et al., 2001; Santos et al., 2007], is totally consistent with the observed pattern of activation of the aforementioned signaling mechanisms by osteostatin in these cells.

Several osteogenic factors are known to interact with the VEGF system as a key mechanism to modulate osteoblastic function [Deckers et al., 2000; Peng et al., 2005; de Gortázar et al., 2006; Samee et al., 2008; Tang et al., 2012; Chen et al., 2012b]. A variety of previous reports have also shown that VEGFR2 activation occurs associated with an increased cell viability and/or proliferation in endothelial and osteoblastic cells [Gerber et al., 1998; Santos and Dias, 2004; Fujita et al., 2006; Santos et al., 2007; Alonso et al., 2008; Liu and Agarwal, 2010]. The results herein demonstrate that Src-mediated VEGFR2 transactivation—leading to ERK and Akt phosphorylation—is a key event associated with several actions of osteostatin, namely promoting VEGF and OPG gene expression and cell survival in osteoblastic cells. Of interest in this regard, osteostatin and fibroblast growth factor-2 were recently found to cooperate in inducing VEGF and its receptors VEGFR1 and VEGFR2 to promote mineralization in MC3T3-E1 cells; and this induction was abolished by an ERK inhibitor [Lozano et al., 2012b]. Moreover, ERK signaling has previously been shown to mediate VEGFR2 gene induction by PTHrP (107–139) in human osteoblastic cells [de Gortázar et al., 2006]. These aggregated findings suggest that interaction of osteostatin with a complex signalsome—including Src, ERK and PI3K/Akt—may represent a positive feed-back mechanism to promote VEGFR2 activation and thus modulate osteoblastic function.

In conclusion, our data demonstrate for the first time the importance of Src activation in the mechanism whereby osteostatin transactivates VEGFR2 to elicit various osteoblastic actions. These findings also provide a new rationale supporting the role of the osteostatin epitope in the C-terminal region of PTHrP as a promoter of bone formation.

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REFERENCES

Alonso V, de Gortázar AR, Ardura JA, Andrade-Zapata I, Alvarez-Arroyo MV, Esbrit P. 2008. Parathyroid hormone-related protein (107–139) increases human osteoblastic cell survival by activation of vascular endothelial growth factor receptor-2. J Cell Physiol 217:717–727.

Ardura JA, Rayego-Mateos S, Rámila D, Ruiz-Ortega M, Esbrit P. 2010. Parathyroid hormone-related protein promotes epithelial-mesenchymal transition. J Am Soc Nephrol 21:237–248.

Bellido T, Ali AA, Plotkin LI, Fu Q, Gubrij I, Roberson PK, Weinstein RS, O'Brien CA, Manolagas SC, Jilka RL. 2003. Proteasomal degradation of Runx2 shortens parathyroid hormone-induced anti-apoptotic signaling in osteoblasts. J Biol Chem 278:50259–50272.

Biscardi JS, Maa MC, Tice DA, Cox ME, Leu TH, Parsons SJ. 1999. c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. J Biol Chem 274:8335–8343.

Boyce BF, Xing L. 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Arch Biochem Biophys 473:139–146.

Bromann PA, Korkaya H, Courtneidge SA. 2004. The interplay between Src family kinases and receptor tyrosine kinases. Oncogene 23:7957–7968.

Chen HT, Tsou HK, Chang CH, Tang CH. 2012a. Hepatocyte growth factor increases osteopontin expression in human osteoblasts through PI3K, Akt, c-Src, and AP-1 signaling pathway. PLoS ONE 7:e38378.

Chen D, Tian W, Li Y, Tang W, Zhang C. 2012b. Osteoblast-specific transcription factor Osterix (Osx) and HIF-1α cooperatively regulates gene expression of vascular endothelial growth factor (VEGF). Biochem Biophys Res Commun 424:176–181.

Chou MT, Wang J, Fujita DJ. 2002. Src kinase becomes preferentially associated with the VEGFR, KDR/Flk-1, following VEGF stimulation of vascular endothelial cells. BMC Biochem 3:32.

Datta NS, Abou-Samra AB. 2009. PTH and PTHrP signaling in osteoblasts. Cell Signal 21:1245–1254.

Dayanir V, Meyer RD, Lashkari K, Rahimi N. 2001. Identification of tyrosine residues in vascular endothelial growth factor receptor-2/FLK-1 involved in activation of phosphatidylinositol 3-kinase and cell proliferation. J Biol Chem 276:17686–17692.

de Castro LF, Lozano D, Dapía S, Portal-Nuñez S, Caeiro JR, Gomez-Barrena E, Esbrit P. 2010. Role of the N- and C-terminal fragments of parathyroid hormone-related protein as putative therapies to improve bone regeneration under high glucocorticoid treatment. Tissue Eng Part A 16:1157–1168.

de Castro LF, Lozano D, Portal-Núñez S, Maycas M, De la Fuente M, Caeiro JR, Esbrit P. 2012. Comparison of the skeletal effects induced by daily administration of PTHrP (1–36) and PTHrP (107–139) to ovariectomized mice. J Cell Physiol 227:1752–1760.

Deckers MM, Karperien M, van der Bent C, Yamashita T, Papapoulos SE, Löwik CW. 2000. Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology 141:1667–1674.

de Gortázar AR, Alonso V, Alvarez-Arroyo MV, Esbrit P. 2006. Transient exposure to PTHrP (107–139) exerts anabolic effects through vascular endothelial growth factor receptor 2 in human osteoblastic cells in vitro. Calcif Tissue Int 79:360–369.

de Miguel F, Fiaschi-Taesch N, López-Talavera JC, Takane KK, Massfelder T, Helwig JJ, Stewart AF. 2001. The C-terminal region of PTHrP, in addition to the nuclear localization signal, is essential for the intracrine stimulation of proliferation in vascular smooth muscle cells. Endocrinology 142:4096–4105.

Dougher M, Terman BI. 1999. Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. Oncogene 18:1619–1627.

Dougher-Vermazen M, Hulmes JD, Böhlen P, Terman BI. 1994. Biological activity and phosphorylation sites of the bacterially expressed cytosolic domain of the KDR VEGF-receptor. Biochem Biophys Res Commun 205:728–738.

Esbrit P, Alvarez-Arroyo MV, de Miguel F, Martín O, Martínez ME, Caramelo C. 2000. C-terminal parathyroid hormone-related protein increases vascular endothelial growth factor in human osteoblastic cells. J Am Soc Nephrol 11:1085–1092.

Fujita Y, Yoshizumi M, Izawa Y, Ali N, Ohnishi H, Kanematsu Y, Ishizawa K, Tsuchiya K, Tamaki T. 2006. Transactivation of fetal liver kinase-1/kinase-insert domain-containing receptor by lysophosphatidylcholine induces vascular endothelial cell proliferation. Endocrinology 147:1377–1385.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N. 1998. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273:30336–30343.

Hobbs SS, Goettel JA, Liang D, Yan F, Edelblum KL, Frey MR, Mullane MT, Polk DB. 2011. TNF transactivation of EGFR stimulates cytoprotective COX-2 expression in gastrointestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 301:G220–G229.

Jin ZG, Ueba H, Tanimoto T, Lungu AO, Frame MD, Berk BC. 2003. Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. Circ Res 93:354–363.

Katz S, Ayala V, Santillán G, Boland R. 2011. Activation of the PI3K/Akt signaling pathway through $P2Y_2$ receptors by extracellular ATP is involved in osteoblastic cell proliferation. Arch Biochem Biophys 513:144–152.

Kendall RL, Rutledge RZ, Mao X, Tebben AJ, Hungate RW, Thomas KA. 1999. Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. J Biol Chem 274:6453–6460.

Koch S, Claesson-Welsh L. 2012. Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med 2:a006502.

Kroll J, Waltenberger J. 1997. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J Biol Chem 272:32521–32527.

Lausson S, Cressent M. 2011. Signal transduction pathways mediating the effect of adrenomedullin on osteoblast survival. J Cell Biochem 112:3807–3815.

Liu J, Agarwal S. 2010. Mechanical signals activate vascular endothelial growth factor receptor-2 to upregulate endothelial cell proliferation during inflammation. J Immunol 185:1215–1221.

Lozano D, Manzano M, Doadrio JC, Salinas AJ, Vallet-Regí M, Gómez-Barrena E, Esbrit P. 2010. Osteostatin-loaded bioceramics stimulate osteoblastic growth and differentiation. Acta Biomater 6:797–803.

Lozano D, Fernández-de-Castro L, Portal-Núñez S, López-Herradón A, Dapía S, Gómez-Barrena E, Esbrit P. 2011. The C-terminal fragment of parathyroid hormone-related peptide promotes bone formation in diabetic mice with low turnover osteopenia. Br J Pharmacol 162:1424–1438.

Lozano D, Trejo CG, Gómez-Barrena E, Manzano M, Doadrio JC, Salinas AJ, Vallet-Regí M, García-Honduvilla N, Esbrit P, Buján J. 2012a. Osteostatin-loaded onto mesoporous ceramics improves the early phase of bone regeneration in a rabbit osteopenia model. Acta Biomater 8:2317–2323.

Lozano D, Feito MJ, Portal-Núñez S, Lozano RM, Matesanz MC, Serrano MC, Vallet-Regí M, Portolés MT, Esbrit P. 2012b. Osteostatin improves the osteogenic activity of fibroblast growth factor-2 immobilized on Si-doped hydroxyapatite in osteoblastic cells. Acta Biomater 8:2770–2777.

Marzia M, Sims NA, Voit S, Migliaccio S, Taranta A, Bernardini S, Faraggiana T, Yoneda T, Mundy GR, Boyce BF, Baron R, Teti A. Decreased c-Src expression enhances osteoblast differentiation and bone formation. J Cell Biol 151:311–320.

Matsumoto T, Claesson-Welsh L. 2001. VEGF receptor signal transduction. Sci STKE 112:re21.

Matsumoto T, Mugishima H. 2006. Signal transduction via vascular endothelial growth factor (VEGF) receptors and their roles in atherogenesis. J Atheroscler Thromb 13:130–135.

Miao D, Su H, He B, Gao J, Xia Q, Zhu M, Gu Z, Goltzman D, Karaplis AC. 2008. Severe growth retardation and early lethality in mice lacking the nuclear localization sequence and C-terminus of PTH-related protein. Proc Natl Acad Sci USA 105:20309–20311.

Pedram A, Razandi M, Levin ER. 1998. Extracellular signal-regulated protein kinase/Jun kinase cross-talk underlies vascular endothelial cell growth factor-induced endothelial cell proliferation. J Biol Chem 273: 26722–26728.

Peng H, Usas A, Olshanski A, Ho AM, Gearhart B, Cooper GM, Huard J. 2005. VEGF improves, whereas sFlt1 inhibits, BMP2-induced bone formation and bone healing through modulation of angiogenesis. J Bone Miner Res 20: 2017–2027.

Petreaca ML, Yao M, Liu Y, DeFea K, Martins-Green M. 2007. Transactivation of vascular endothelial growth factor receptor-2 (VEGFR2) by interleukin-8 (IL-8/CXCL8) is required for IL-8/CXCL8-induced endothelial permeability. Mol Biol Cell 18:5014–5023.

Portal-Núñez S, Lozano D, Esbrit P. 2012. Role of angiogenesis in bone formation. Histol Histopathol 27:559–566.

Roskoski R, Jr. 2004. Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun 324:1155–1164.

Samee M, Kasugai S, Kondo H, Ohya K, Shimokawa H, Kuroda S. 2008. Bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) transfection to human periosteal cells enhances osteoblast differentiation and bone formation. J Pharmacol Sci 108:18–31.

Santos SC, Dias S. 2004. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. Blood 103:3883–3889.

Santos SC, Miguel C, Domingues I, Calado A, Zhu Z, Wu Y, Dias S. 2007. VEGF and VEGFR-2 (KDR) internalization is required for endothelial recovery during wound healing. Exp Cell Res 313:1561–1574.

Schipani E, Maes C, Carmeliet G, Semenza GL. 2009. Regulation of osteogenesis-angiogenesis coupling by HIFs and VEGF. J Bone Miner Res 24: 1347–1353.

Takahashi T, Yamaguchi S, Chida K, Shibuya M. 2001. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. EMBO J 20:2768–2778.

Tang W, Yang F, Li Y, de Crombrugghe B, Jiao H, Xiao G, Zhang C. 2012. Transcriptional regulation of vascular endothelial growth factor (VEGF) by osteoblast-specific transcription factor osterix (Osx) in osteoblasts. J Biol Chem 287:1671–1678.

Tanimoto T, Jin ZG, Berk BC. 2002. Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine

1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS). J Biol Chem 277:42997–43001.

Thomas SM, Brugge JS. 1997. Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol 13:513–609.

Toribio RE, Brown HA, Novince CM, Marlow B, Hernon K, Lanigan LG, Hildreth BE III, Werbeck JL, Shu ST, Lorch G, Carlton M, Foley J, Boyaka P, McCauley LK, Rosol TJ. 2010. The midregion, nuclear localization sequence, and C terminus of PTHrP regulate skeletal development, hematopoiesis, and survival in mice. FASEB J 24:1947–1957.

Trejo CG, Lozano D, Manzano M, Doadrio JC, Salinas AJ, Dapía S, Gómez-Barrena E, Vallet-Regí M, García-Honduvilla N, Buján J, Esbrit P. 2010. The osteoinductive properties of mesoporous silicate coated with osteostatin in a rabbit femur cavity defect model. Biomaterials 31:8564–8573.

Valín A, Guillén C, Esbrit P. 2001. C-terminal parathyroid hormone-related protein (PTHrP) (107–139) stimulates intracellular Ca^{2+} through a receptor different from the type 1 PTH/PTHrP receptor in osteoblastic osteosarcoma UMR 106 cells. Endocrinology 142:2752–2759.

Zachary I, Gliki G. 2001. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc Res 49:568–581.

Zeng H, Sanyal S, Mukhopadhyay D. 2001. Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. J Biol Chem 276: 32714–32719.

Zhang R, Xu Y, Ekman N, Wu Z, Wu J, Alitalo K, Min W. 2003. Etk/Bmx transactivates vascular endothelial growth factor 2 and recruits phosphatidylinositol 3-kinase to mediate the tumor necrosis factor-induced angiogenic pathway. J Biol Chem 278:51267–51276.

Zilberberg L, Shinkaruk S, Lequin O, Rousseau B, Hagedorn M, Costa F, Caronzolo D, Balke M, Canron X, Convert O, Lain G, Gionnet K, Gonçalves M, Bayle M, Bello L, Chassaing G, Deleris G, Bikfalvi A. 2003. Structure and inhibitory effects on angiogenesis and tumor development of a new vascular endothelial growth inhibitor. J Biol Chem 278:35564–35573.