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EGFR trans-activation mediates pleiotrophin-induced activation of Akt and Erk in cultured osteoblasts

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ABSTRACT

Pleiotrophin (Ptn) plays an important role in bone growth through regulating osteoblasts' functions. The underlying signaling mechanisms are not fully understood. In the current study, we found that Ptn induced heparin-binding epidermal growth factor (HB-EGF) release to trans-activate EGF-receptor (EGFR) in both primary osteoblasts and osteoblast-like MC3T3-E1 cells. Meanwhile, Ptn activated Akt and Erk signalings in cultured osteoblasts. The EGFR inhibitor AG1478 as well as the monoclonal antibody against HB-EGF (anti-HB-EGF) significantly inhibited Ptn-induced EGFR activation and Akt and Erk phosphorylations in MC3T3-E1 cells and primary osteoblasts. Further, EGFR siRNA depletion or dominant negative mutation suppressed also Akt and Erk activation in MC3T3-E1 cells. Finally, we observed that Ptn increased alkaline phosphatase (ALP) activity and inhibited dexamethasone (Dex)-induced cell death in both MC3T3-E1 cells and primary osteoblasts, such effects were alleviated by AG1478 or anti-HB-EGF. Together, these results suggest that Ptn-induced Akt/Erk activation and some of its pleiotropic functions are mediated by EGFR trans-activation in cultured osteoblasts.

1. Introduction

In the bone matrix, a number of osteo-inductive factors have the potential to stimulate bone growth. Of these, the 136 amino acid bone growth factor pleiotrophin (Ptn) plays an important role in bone growth and formation [1–5]. Ptn, also known as osteoblast stimulating factor 1 (OSF-1) or heparin-binding growth-associated molecule (HB-GAM), is highly conserved with more than 90% similarity across species [1–3]. It shares 50% similarity with the midkine (MK) protein [1–3]. Ptn is highly expressed in bone (also in the brain) during embryonic development, it is down-regulated in postnatal growth of bone, and its expression is ceased after skeletal maturity [1,6].

Ptn is an important matrix-bound osteo-growth factor. It serves as anchemotactic signaling molecule to promote migration of osteoblasts and osteoblast precursors [7]. Yang et al. showed that Ptn could enhance the attachment and subsequent differentiation of MC3T3-E1 cells. In the cultured osteoblasts, Ptn has been shown to promote osteoblast proliferation, migration and differentiation [8]. Increasing cell motility (migration) is another possible function of Ptn [8]. Ptn overexpressing mice demonstrated an increased bone mineral content (BMC). The transgenic mice had relatively longer tibiae and femurs than the age-matched wild-type mice, indicating that Ptn in the bone tissues could promote bone growth [4,9].

The pleiotropic functions of Ptn are thought to be regulated primarily in an autocrine/paracrine manner, and is mediated through receptor-activated signaling [1]. So far, at least five Ptn receptors have been indentified, including receptor protein-tyrosine phosphatase β/ζ (RPTP β/ζ), anaplastic lymphoma kinase (ALK), syndecan-1, syndecan-3, syndecan-4 [1,10,11]. Ptn-receptor binding has been shown to activate multiple signaling pathways including the Ras-mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways [1,10,11], which promotes cell growth, cell survival and inhibits programmed cell death (apoptosis) [12,13]. However, the underlying mechanism of Erk and Akt activation by Ptn, particularly in osteoblasts, is still not fully understood.

The epidermal growth factor (EGF)-receptor (EGFR) signaling network is one of the best-characterized signaling systems [14]. EGFR and their ligands modulate cellular functions in a variety of ways, including proliferation, survival, adhesion, migration, and differentiation [15]. The EGFR ligands, including EGF, amphiregulin, and transforming growth factor alpha (TGFα) as well as and heparin binding EGF (HB-EGF) and epiregulin, bind to and activate

Abbreviations: Ptn, pleiotrophin; ALP, alkaline phosphatase; EGF, epidermal growth factor; HB-EGF, heparin-binding-epidermal growth factor; Dex, dexamethasone.

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EGFR [15]. Activated EGFR recruits adaptor proteins to activate downstream signaling pathways including PI3K-Akt, Erk/MAPK and PLC axis [16], eventually promoting cell survival, migration, proliferation and inhibiting cell apoptosis [15]. Besides being directly stimulated by its ligands, EGFR could also be activated indirectly by a number of stimuli, a process also known as "trans-activation" [17–21]. In the current study, we found that Ptn-induced Erk and Akt activation and some of its pleiotropic functions were also dependent on EGFR trans-activation in osteoblasts.

2. Material and methods

2.1. Chemical and reagents

AG-1478 as well as recombinant ("r") mouse Ptn (rPtn), EGF (rEGF) and HB-EGF (r-HB-EGF) were purchased from Calbiochem (Shanghai, China).

2.2. Cell culture

The murine calvaria-derived osteoblastic MC3T3-E1 cell line, a gift from Dr. Xiao-dong Wang [22], was seeded at 1×10^5 cells/ml into 75-cm^2 flasks and maintained in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin. This basic medium was replenished every 2–3 days. The cultures were then induced to differentiate by transferring cells into the medium supplemented with L-ascorbic acid (50 μg/ml, Sigma) and β-glycerol phosphate (5 mM, Sigma).

2.3. Primary osteoblasts isolation and culture

As previously described [22], murine osteoblasts were isolated from pups at 30–36 h old. The animals were sedated by hypothermia and then killed by decapitation. The calvariae were removed and bathed in α-MEM. The fibrous tissue surrounding the bone was gently scraped, and sutures were removed. The trimmed calvariae were transferred to a 50-ml Erlenmeyer flask and washed with PBS three times, at 37 °C for 10 min per wash, in an oscillating water bath. Calvariae were then subjected to a series of collagenase digestions in an oscillating 37 °C water bath. The first two digests were discarded. Digests 3, 4, and 5 were neutralized with α-MEM, pooled, and filtered through sterile polypropylene mesh of 200–297 μl. The filtrate was centrifuged for 6 min at 1500 rpm, the supernatant was removed, and cells were resuspended in 3–5 ml α-MEM containing 10% FBS with antibiotics. Cells were counted and cultured until reaching confluence at 7–9 days, medium was switched every 2–3 days. All animals were maintained in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996), the European Communities Council. The protocol is approved by Animal Care and Use Committee of all authors' institutions.

2.4. Western blots

After treatment, cell lysates were extracted from osteoblasts with a RIPA lysis buffer. Protein concentration was determined by Bio-Rad protein concentration assay. Aliquots of 30–40 μg of lysates were electrophoresed on 10% SDS–PAGE gel and transferred to PVDF membranes. The blots were then incubated with primary antibodies at 4 °C overnight. The appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) were then added. The antigen–antibody complex was detected by using enhanced chemiluminescence (ECL) reagent. Each condition was carried out in triplicate. The intensity of indicated band was quantified by densitometry using ImageJ software, and was normalized to non-phosphorylated kinase. Quantification value was expressed as the fold change vs. the band labeled with "1.0". ImageJ was downloaded from NIH website (http://rsbweb.nih.gov/ij/download.html).

2.5. Antibodies

The antibodies against phospho-EGFR (Tyr 1068, Tyr 1045), EGFR, phospho-Erk1/2 (pThr202/Tyr204), Erk1/2, phospho-Akt (pSer473) and Akt were from purchased from Cell Signaling Technology (Beverly, MA). The monoclonal anti-HB-EGF was purchased from Calbiochem (Shanghai, China).

2.6. EGFR siRNA knockdown

Two non-overlapping siRNAs against mouse EGFR and a negative control scramble siRNA were purchased from Dharmacon Research Inc. (Lafayette, CO, USA). We applied siRNA duplexes at a final concentration of 200 nM using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). The transfection took 48 h, and expression of target protein (EGFR) was always tested by Western blots.

2.7. EGFR dominant negative mutation

EGFR-CD533, a truncated EGFR mutant missing the COOH-terminal 533 amino acids, which lacks mitogenic and transformation activity, was amplified by PCR using 5′-GCATCATCT AGAGCCACCATGCGACCCTCCGGG-3′ as forward and 5′-GCATCACTC GAGTCAGCGCTTCCGAACGATG-3′ as reverse primer [23,24]. The PCR product was designed to insert XbaI andXhoI restriction sites, and was ligated into the p-Super-puro-eGFP vector. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was used to transfect the plasmid or the empty vector (2 μg/well) into cells according the manufacturer's protocol. Stable clones were selected by puromycin (0.5 μg/ml). The cell culture medium was replaced with fresh puromycin-containing medium every 2–3 days, until resistant colonies can be identified (2 weeks). The EGFR expression was always detected by Western blots in the resistant colonies.

2.8. Cell viability assay

As previously reported [25], cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates at a density of $1–2 \times 10^4$ cells/well. Twenty-four hours after the indicated stimulation, MTT tetrazolium salt (Sigma, 0.20 mg/ml) was added to each well, cells were further incubated in CO_2 incubator for 2–3 h. DMSO (200 μl/well) was then added to dissolve formazan crystals, the absorbance of each well was observed by a plate reader at a wavelength of 490 nm. For each treatment, $n = 6$. OD number of treatment group was normalized to that of control group.

2.9. Cell death detection

The osteoblasts were seeded subconfluently into 12-well plates and treated with conditioned medium containing indicated drugs for 24 h. Cells were trypsinized and pelleted with cellular supernatant for 5 min at 400*g*. After pellet was resuspended in 60 μL media, cell death percentages were determined by counting cells using a hemocytometer after addition of trypan blue, which stained the cytoplasm of dead cells. Cell death percentage (%) = the number of trypan blue stained cells/the number of total cells (×100%).

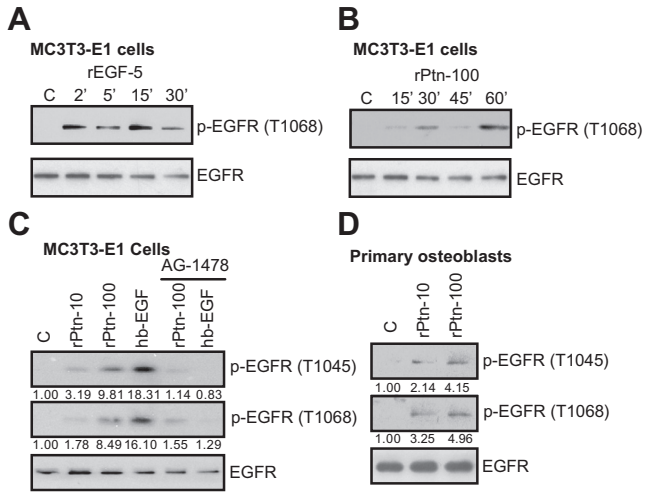

Fig. 1. Ptn activates EGFR in osteoblasts. MC3T3-E1 osteoblastic cells were either left untreated ("C"), or treated with recombinant (r) mouse EGF (5 ng/ml) (A) or rPtn (100 ng/ml) (B) for indicated time, p-EGFR (Tyr 1068) and total-EGFR were tested by Western blots. The MC3T3-E1 cells were pre-treated with EGFR inhibitor AG-1478 (1 μM) for 1 h, followed by rPtn (10/100 ng/ml, 1 h) or HB-EGF (5 ng/ml, 2 min) stimulation, p-EGFR (Tyr 1068, Tyr 1045) and regular EGFR were tested (C). The primary osteoblasts were treated with rPtn (10/100 ng/ml) for 1 h, p-EGFR (Tyr 1068, Tyr 1045) and total EGFR were tested (D). Experiments were repeated for three times, and similar results were obtained.

2.10. Alkaline phosphotase (ALP) activity assay

After treatment, alkaline phosphatase (ALP) activity in osteoblasts was determined by detecting the formation of *p*-nitrophenol, a product of *p*-nitrophenyl phosphate catalyzed by ALP, according to a previously described colorimetric procedure provided by a Sigma Diagnostics Alkaline, Acid, and Prostatic Acid Phosphotase kit (Sigma) [26].

2.11. Statistical analyses

All experiments were repeated at least three times, and similar results were obtained. Data were expressed as mean ± SD. Statistical analyses were analyzed by one-way analysis of variance using GraphPad InStat version 3. Multiple comparisons were performed using Tukey's honestly significant difference procedure. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Ptn activates EGFR in osteoblasts

In the current study, we are interested to know the potential role of EGFR in Ptn signaling in osteoblasts. As expected, in MC3T3-E1 osteoblastic cells, mouse EGF (5 ng/ml) induced an early, strong and sustained EGFR activation, which was confirmed by upregulation of p-EGFR (Tyr 1068) (Fig. 1A). Significantly, using the same method, we observed EGFR activation by Ptn (100 ng/ml). Note that Ptn-induced EGFR phosphorylation was lower, and came later (Fig. 1B). AG-1478, the known EGFR inhibitor [27], blocked Ptn-induced EGFR phosphorylations at Tyr-1045 and Tyr-1068 (Fig. 1C). HB-EGF-induced EGFR activation was also blocked by AG-1478 (Fig. 1C). Further, Ptn activated EGFR in primary mouse osteoblasts, as p-EGFR (Tyr-1045 and Tyr-1068) was significantly up-regulated after Ptn stimulation (Fig. 1D).

3.2. Ptn induces HB-EGF release in osteoblasts

The above results showed that Ptn activated EGFR in osteoblasts, next we explored the potential mechanism by focusing on

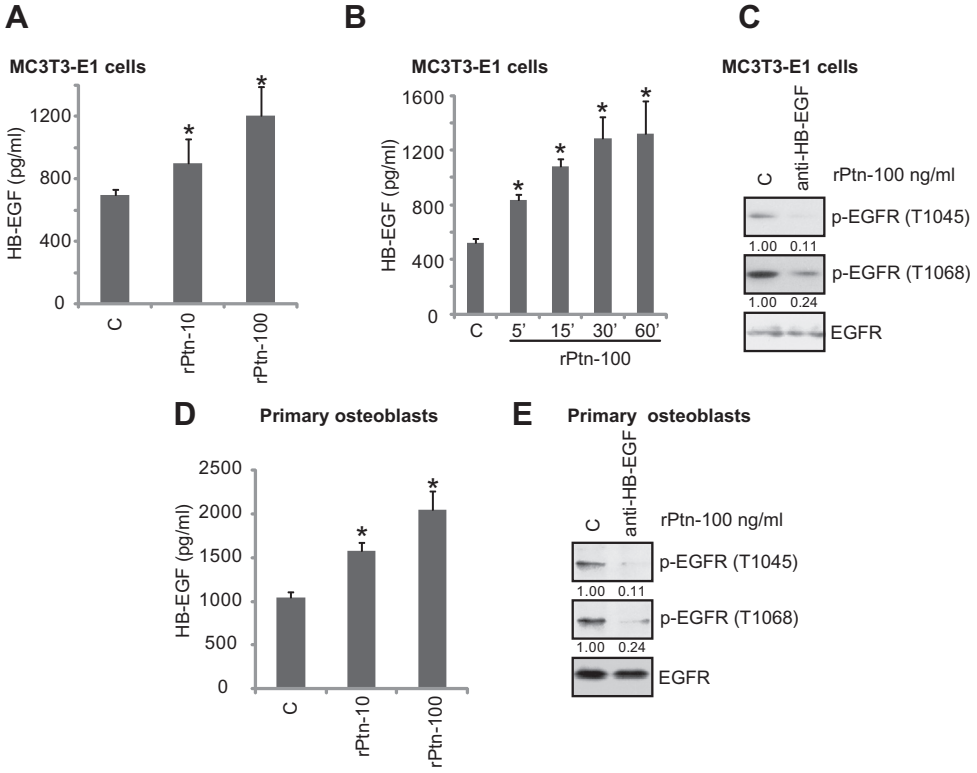

Fig. 2. Ptn induces HB-EGF release in osteoblasts. MC3T3-E1 cells and primary osteoblasts were either left untreated ("C"), or treated with rPtn (10/100 ng/ml) for indicated time, HB-EGF content in the cultured medium was analyzed by ELISA assay (A, B and D). MC3T3-E1 cells and primary osteoblasts were pretreated with anti-HB-EGF (500 μg/ml) for 1 h, followed by rPtn (100 ng/ml, 1 h) stimulation, p-EGFR (Tyr 1068, Tyr 1045) and total EGFR were tested (C and E). Experiments were repeated for three times, and similar results were obtained. $p < 0.05$ vs. group "C" (A, B and D).

HB-EGF. ELISA results in Fig. 2A and B showed that the content of HB-EGF in the condition medium of Ptn-stimulated MC3T3-E1 cells was significantly increased. The effect of Ptn on HB-EGF release was both dose- and time-dependent (Fig. 2A and B). HB-EGF content went up as early as 5 min after Ptn stimulation, and lasted at least for 1 h (Fig. 2B). Significantly, the monoclonal antibody against HB-EGF (anti-HB-EGF) significantly inhibited Ptn-induced EGFR phosphorylation at both Tyr-1045 and Tyr-1068 (Fig. 2C), indicating that HB-EGF release might mediate EGFR trans-activation by Ptn. In primary osteoblasts, Ptn similarly induced HB-EGF release (Fig. 2D), and anti-HB-EGF suppressed Ptn-induced EGFR activation (Fig. 2E).

3.3. Ptn-induced Akt and Erk activation requires EGFR activation in osteoblasts

Next we explored the role of EGFR in Ptn signaling in cultured osteoblasts. We first examined Akt and Erk activation by Ptn through Western blot using the phosphorylated (p)-antibodies. As shown in Fig. 3A, Ptn-induced significantly Akt and Erk activation in MC3T3-E1 cells, as p-Akt and p-Erk went up after Ptn stimulation (Fig. 3A). Importantly, EGFR inhibitor AG-1478 and anti-HB-EGF dramatically inhibited Ptn-induced Akt and Erk activation in MC3T3-E1 cells (Fig. 3B). EGFR silencing by targeted siRNAs (we used two non-overlapping siRNAs) as well as EGFR dominant negative mutation (CD533) [23,24] largely inhibited Akt/Erk activation by Ptn in MC3T3-E1 cells (Fig. 3C and D). These results suggest that EGFR activation is required for Akt/Erk activation by Ptn in osteoblasts. Further, Ptn-induced Akt and Erk activation in primary osteoblasts was also inhibited by AG-1478 and anti-HB-EGF (Fig. 3E).

3.4. EGFR activation is important for Ptn-induced increase of alkaline phosphatase (ALP) activity and cell survival in osteoblasts

It has been shown that Ptn increased ALP activity in cultured MC3T3-E1 cells [28]. We thus examined the potential role of EGFR in Ptn's effect. Results showed that Ptn-induced ALP activity increase was inhibited by AG-1478 and anti-HB-EGF in MC3T3-E1 cells and primary osteoblasts (Fig. 4A and C). Meanwhile, EGFR-siRNA knockdown or dominant negative mutation largely inhibited ALP activity enhancement by Ptn (Fig. 4B). These results suggest that EGFR activation is required for Ptn-induced ALP increase in osteoblasts. Since both Akt and Erk are important survival signalings, we then tested the potential role of Ptn in osteoblast cell survival. As shown in Fig. 4A and C, in both MC3T3-E1 cells and primary osteoblasts, dexamethasone (Dex) inhibited cell survival, which was alleviated by Ptn co-administration (Fig. 4D–F). Significantly, AG-1478 and anti-HB-EGF inhibited Ptn-induced pro-survival effect against Dex (Fig. 4D–F). These results suggested that EGFR activation is also important for Ptn-induced osteoblast cell survival.

4. Discussions

Studies have shown addition of Ptn to cultures of MC3T3-E1 cells could enhance ALP activity and cell growth [28]. Meanwhile, Ptn could also increase osteoblast cell chemo-taxis [29]. Consistent with these studies, we found that Ptn activated Akt and Erk signalings, increased ALP activity and promoted cell survival (against Dex) in both primary osteoblasts and osteoblast-like MC3T3-E1 cells. Significantly, our data showed that HB-EGF release and subsequent EGFR trans-activation might be the key signaling molecular event for Ptn-induced signaling activation and its pleiotropic functions, as EGFR inhibitor AG1478 and anti-HB-EGF largely inhibited Ptn-induced Akt/Erk activation, ALP activity increase and its pro-survival effect.

Ptn has pro-growth and pro-survival (anti-apoptosis) effects on a variety of normal cell types and tumor cells [12,13,26,30–32], however, there is little published data illustrating the signaling mechanisms involved in Ptn's effect, particularly in the osteoblasts. It has been shown that Ptn rescues both SW-13 and NIH3T3 fibroblasts from serum starvation-induced apoptosis, and the effect of Ptn is mediated through the ALK receptor–dependent Akt and Erk activation. However, this is unlikely the case here in the osteoblasts. First, there is no reports so far showing ALK expression in osteoblasts (We also failed to detect ALK expression in osteoblasts). Second, crizotinib (PF02341066), the ALK inhibitor, had no effect on Ptn-induced Akt/Erk activation or pro-survival effect in osteoblasts (Data not shown). Here, our data suggest that EGFR trans-activation might be the key mediator for Ptn-induced Akt/Erk activation.

Dex and other glucocorticoids (GCs) are commonly used anti-inflammatory and immuno-suppressive drugs to treat local/

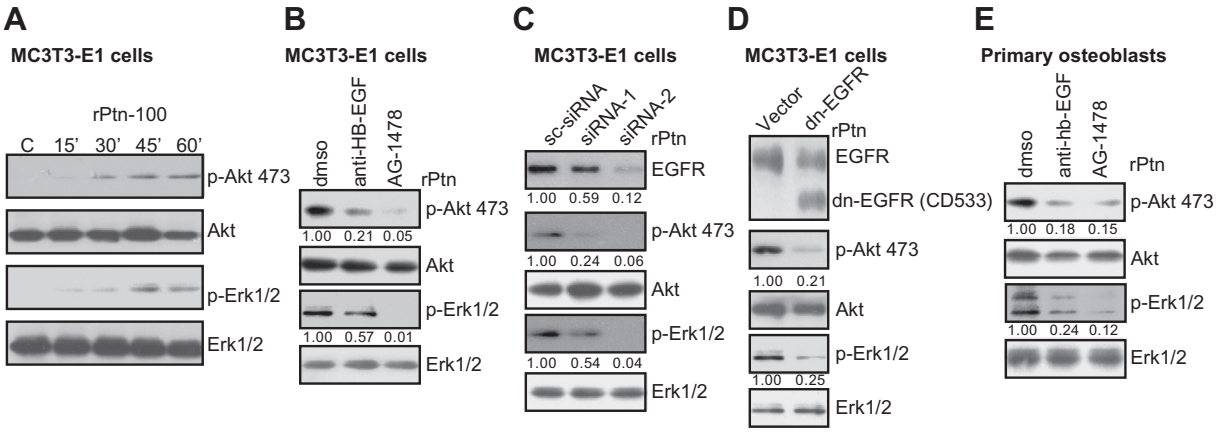

Fig. 3. Ptn-induced Akt and Erk activation requires EGFR activation in osteoblasts. MC3T3-E1 cells were either left untreated ("C"), or treated with rPtn (100 ng/ml) for indicated time, p-/total-Akt and Erk1/2 were tested by Western blots (A). MC3T3-E1 cells and primary osteoblasts were pretreated with AG-1478 (1 μM) or anti-HB-EGF (500 μg/ml) for 1 h, followed by rPtn (100 ng/ml) stimulation, p-/total-Akt and Erk1/2 were tested (B and E). MC3T3-E1 cells transfected with scramble siRNA (sc-siRNA), or EGFR siRNA (two non-overlapping siRNAs) (200 nM each, 48 h) were treated with rPtn (100 ng/ml) for 1 h, EGFR expression as well as p-/total-Akt and Erk1/2 were tested (C). Stable MC3T3-E1 cells transfected with vector control (p-Super-puro-eGFP) or the dominant negative EGFR (p-Super-EGFR-CD533-puro-eGFP) (2 μg/ml each) were treated with rPtn (100 ng/ml) for 1 h, EGFR as well as p-/total-Akt and Erk1/2 were tested (D). Experiments were repeated for three times, and similar results were obtained.

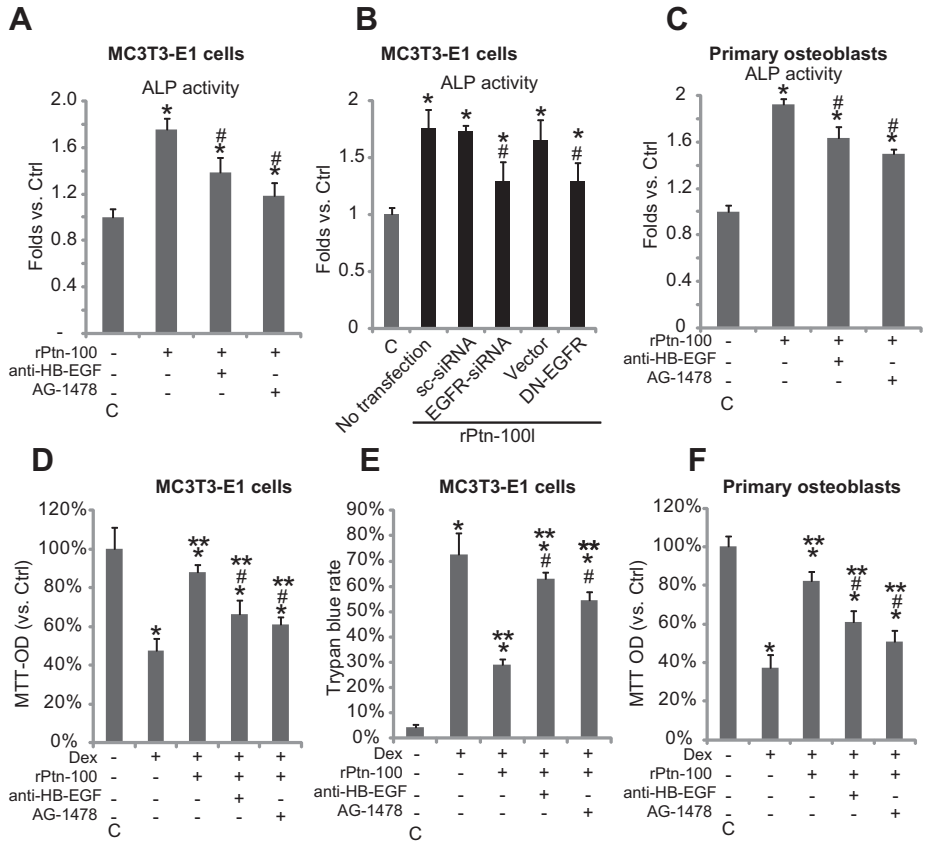

Fig. 4. EGFR activation is important for Ptn-induced increase of alkaline phosphatase (ALP) activity and cell survival in osteoblasts. MC3T3-E1 cells and primary osteoblasts were either left untreated ("C"), or treated with rPtn (100 ng/ml) in the presence or absence of AG-1478 (1 μM) or anti-HB-EGF (500 μg/ml) for 24 h, ALP activity was examined (A and C). MC3T3-E1 cells transfected with scramble/EGFR siRNA (200 nM each), or with empty vector/dominant negative EGFR (2 μg/ml) were treated with rPtn (100 ng/ml), ALP activity was analyzed (B). MC3T3-E1 cells and primary osteoblasts were either left untreated ("C"), or stimulated with Dex (1 μM), Dex + rPtn (100 ng/ml), Dex + rPtn + AG-1478 (1 μM) or Dex + anti-HB-EGF (500 μg/ml) for 24 h, cell survival and death was analyzed by MTT assay (D and F) and trypan blue staining assay (E) respectively. Experiments were repeated for three times, and similar results were obtained. $^{*}p < 0.05$ vs. "C" group (A–F), $^{\#}p < 0.05$ vs. "rPtn-100" group (A–F). $^{**}p < 0.05$ vs. Dex group (D–F).

systemic inflammations, autoimmune disorders and other disease [33]. However, prolonged and/or overdose GCs usage is known to cause secondary osteoporosis [34,35] and osteonecrosis [36]. More than 30% of patients receiving long-term GCs therapy have bone fractures [34]. These patients have fewer osteoblasts and increased osteocytes' apoptosis in their bone [34,35].The fewer number of osteoblasts in these osteoporosis/osteonecrosis patients is mainly due to decreased osteoblastogenesis, decreased osteoblast differentiation, and more importantly, an increased level of osteoblast death, together causing reduction in bone strength and formation [34,35]. In the current study, we found that Ptn administration could significantly inhibit Dex-induced osteoblast cell death and viability loss. These results are not surprising, as both Akt and Erk signalings were major pro-survival molecular effectors, and here we found that Ptn induced profound Akt and Erk activation in osteoblasts. In summary, our data suggest that Ptn-induced Erk and Akt activation as well as some of its pleiotropic functions are dependent on EGFR trans-activation in osteoblasts.

Competing interests

The authors declare that they have no competing interests.

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