

# EphB4/ TNFR2/ERK/MAPK signaling pathway comprises a signaling axis to mediate the positive effect of TNF- $\alpha$ on osteogenic differentiation

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## Research article

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# Abstract

**Background :** Low concentrations of tumor necrosis factor-alpha (TNF- $\alpha$ ) and its receptor TNFR2 are both reported to promote osteogenic differentiation of osteoblast precursor cells. Moreover, low concentrations of TNF- $\alpha$  up-regulate the expression of EphB4. However, the molecular mechanisms underlying TNF- $\alpha$ -induced osteogenic differentiation and the roles of TNFR2 and EphB4 have not been fully elucidated.

**Results :** This study showed that with increase in ALP activity, mRNA and protein expression of RUNX2, BSP and EphB4, TNFR2 expression was significantly promoted under 0.5 ng/ml TNF- $\alpha$  stimulation in osteoblast precursor cells MC3T3-E1. After TNFR2 was inhibited by gene knockdown with lentivirus-mediated shRNA interference or by a neutralizing antibody against TNFR2, the pro-osteogenic effect of TNF- $\alpha$  was partly reversed, but up-regulation of EphB4 by TNF- $\alpha$  kept unchanged. With EphB4 forward signaling suppressed by a potent inhibitor of EphB4 auto-phosphorylation, NVP-BHG712, TNF- $\alpha$ -enhanced expressions of TNFR2, BSP and Runx2 were significantly decreased. Further investigation into the signaling pathways revealed that TNF- $\alpha$  significantly augmented expression of p -JNK, p -ERK and p -p38, however, only p -ERK expression was significantly inhibited in TNFR2-knockdown cells. In addition, the ERK pathway inhibitor, U0126 (10 $\mu$ M), significantly inhibited the protein levels of RUNX2 and BSP when compared with cells treated with TNF- $\alpha$  only.

**Conclusions :** The EphB4, TNFR2 and ERK/MAPK signaling pathway comprise a signaling axis to mediate the positive effect of TNF- $\alpha$  on osteogenic differentiation.

# Background

As a pleiotropic cytokine, tumor necrosis factor alpha (TNF- $\alpha$ ) is primarily produced by activated macrophages and lymphocytes, and can also be detected in endothelial cells and other cell types [1]. The biological activities of TNF- $\alpha$  are mediated through two structurally distinct receptors, tumor necrosis factor receptor 1 (TNFR1) and TNFR2. In osteoblast precursor cells, TNFR1 mediates activation of NF- $\kappa$ B signaling and inhibition of osteogenic differentiation after TNF- $\alpha$  stimulation [2], whereas TNFR2 activation by progranulin (PGRN), a TNFR2 agonist, plays a protective role in osteogenic differentiation and bone regeneration [3,4]. Furthermore, TNF- $\alpha$  affects behaviors of mesenchymal stem cells (MSCs) and osteoblast precursor cells in a dose and time-dependent manner [2,5]. Classically, TNF- $\alpha$  is known as an inhibitor of osteoblastic differentiation and an activator of osteoclastogenesis [5,6,7]. However, opposite findings suggest that TNF- $\alpha$  may be an important inducer of osteogenic differentiation and bone healing [8,9]. Interestingly, during the healing processes of bone fracture the expression of TNF- $\alpha$  and its receptors, TNFR1 and TNFR2, presents a biphasic pattern. At the early stage after bone injury is created in mouse models, TNF- $\alpha$  expression is mainly found in macrophages and other inflammatory cells, accompanied by the release of secondary signaling molecules to recruit osteoprogenitor cells via chemotactic effect. At the late stage of wound healing, TNF- $\alpha$  expression is mainly detected in osteoblasts and other mesenchymal cells [10,11], indicating that TNF- $\alpha$  actively participates in bone fracture regeneration [9]. Indeed, according to the literature data and our previous studies, a short-term exposure to low concentrations of TNF- $\alpha$

enhances the osteogenic differentiation of MSCs and osteoblasts, which in turn promotes bone regeneration [12,13,14,15].

Several molecular mechanisms have been reported to play roles in TNF- $\alpha$ -induced osteogenic differentiation [14,15,16]. For example, TNF- $\alpha$  modulates the expressions and functions of the adenosine A2B receptors (A2BARs), which have been shown to promote osteogenic differentiation of MSCs both *in vitro* and *in vivo* [17,18]. Wnt5a and NF- $\kappa$ B were also reported to be involved in TNF- $\alpha$ -induced osteogenic differentiation [19,20,21]. Moreover, we found that ephrinB2-EphB4 signaling pathway, which participates in a broad spectrum of biological processes [22,23,24,25], at least partly mediates the osteogenic differentiation promoted by low concentrations of TNF- $\alpha$  [16]. However, whether there exists the crosstalk between TNFR2 and EphB4 signaling in the pro-osteogenic effect of low concentrations of TNF- $\alpha$  remains to be elucidated.

In this study, pre-osteoblast cells were stimulated with a low concentration of TNF- $\alpha$ , and expression levels of TNFR2, EphB4 and osteogenesis associated markers as well as **corresponding** signaling cascades were investigated. We found for the first time that the EphB4/ TNFR2 and ERK/MAPK signaling axis partly mediates TNF- $\alpha$ -induced osteogenic differentiation of these pre-osteoblast cells.

## Results

### **TNF- $\alpha$ promotes osteogenic differentiation of MC3T3-E1 cells.**

MC3T3-E1 cells were treated with or without 0.5ng/ml TNF- $\alpha$  for 24h or 48h, and subjected to CCK-8 assay. No statistically significant difference in cell proliferation/survival was found when compared the TNF- $\alpha$ -stimulated MC3T3-E1 cells with the control cells (Fig.1a). We then cultured MC3T3-E1 cells in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$  for 7d or 14d, and determined the ALP activity in these cells. Our results indicated that TNF- $\alpha$  treatment significantly increased the ALP activity when compared with the control cells (Fig.1b). We also monitored the mRNA and protein expression levels of RUNX2 and BSP in MC3T3-E1 cells cultured in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$  for 24h or 48h. We found that the expression levels of RUNX2 and BSP were both significantly higher in TNF- $\alpha$ -treated MC3T3-E1 cells than in the control cells (Fig.1c-f).

### **TNF- $\alpha$ promotes the expression of TNFR2 in MC3T3-E1 cells**

MC3T3-E1 cells were treated with or without 0.5ng/ml TNF- $\alpha$  in the regular culture medium for 24h or 48h, and the expression levels of TNFR2 were determined using RT-PCR, western blot and immunofluorescence staining for TNFR2. Compared with those in the control cells, the mRNA and protein levels of TNFR2 in TNF- $\alpha$ -treated cells were both significantly increased (Fig.2a, b). As confirmed by the immunofluorescence staining analysis, cells incubated in the presence of TNF- $\alpha$  emitted stronger red fluorescence than the control cells (Fig. 2c). Similar results were obtained in the MC3T3-E1 cells cultured in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$  for 24h or 48h, indicating that the

expression of TNFR2 was also up-regulated by TNF- $\alpha$  stimulation during the processes of osteogenic differentiation (Fig. 2d-f).

### **EphB4 mediates the promoted TNFR2 expression and pro-osteogenic effect by TNF- $\alpha$**

Our previous study demonstrated that TNF- $\alpha$  significantly enhances EphB4 expression [16]. To investigate the crosstalk of TNFR2 and EphB4 in the pro-osteogenic effect of TNF- $\alpha$ , we knocked down the expression level of TNFR2 in MC3T3-E1 cells with lentivirus-mediated shRNA interference. Totally 3 siRNAs specifically targeting TNFR2 were designed and synthesized, and the sequences of these siRNAs were listed in Table 1. All of these siRNAs were duplexed, ligated into the pHBLV-ZsGreen-Puro expression vector, and packaged into lentiviral particles. The lentiviral particles were named as pHBLV-TNFR2siRNA1, pHBLV-TNFR2siRNA2 and pHBLV-TNFR2siRNA3, respectively. An siRNA with no homology to any known mouse or human gene was also synthesized, duplexed, ligated into the pHBLV-ZsGreen-Puro expression vector and packaged into lentiviral particles to serve as a negative control. The sequence of the negative control siRNA was also listed in Table 2, and the resulting control lentiviral particles were named as pHBLV-NC. MC3T3-E1 cells were then infected with pHBLV-TNFR2siRNA1, pHBLV-TNFR2siRNA2, pHBLV-TNFR2siRNA3 or pHBLV-NC, and the stably transduced cells were selected with puromycin and named as pHBLV-TNFR2siRNA1 cells, pHBLV-TNFR2siRNA2 cells, pHBLV-TNFR2siRNA3 cells and pHBLV-NC cells, respectively.

We first detected the mRNA expression levels of TNFR2 in pHBLV-TNFR2siRNA1 cells, pHBLV-TNFR2siRNA2 cells, pHBLV-TNFR2siRNA3 cells and pHBLV-NC cells and found that the pHBLV-TNFR2siRNA1 cells displayed the highest TNFR2 gene silencing efficiency (Fig. 3a), and thus selected this cell line to continue the following studies. As confirmed by the western blot analysis, pHBLV-TNFR2siRNA1 cells showed an approximately 70% reduction in the TNFR2 protein level when compared with the pHBLV-NC cells (Fig. 3b). To observe the role of TNFR2 in TNF- $\alpha$  enhanced osteogenic differentiation and EphB4 expression, the pHBLV-TNFR2siRNA1 cells and the pHBLV-NC cells were then cultured in the osteogenic induction medium supplemented with 0.5ng/ml TNF- $\alpha$  for 24h or 48h, and the mRNA and protein levels of EphB4, RUNX2 and BSP were determined. Compared with those in the pHBLV-NC cells, expression levels of RUNX2 and BSP were significantly lower in the pHBLV-TNFR2siRNA1 cells. However, no significant difference was detected in the EphB4 expression levels between the pHBLV-TNFR2siRNA1 cells and the pHBLV-NC cells (Fig. 3c-f), suggesting that TNFR2 activation can not affect EphB4 expression in TNF- $\alpha$  activated osteogenic process.

To further confirm these results, the binding of TNF- $\alpha$  to TNFR2 was blocked by an anti-mouse TNFR2/CD120b/TNFRSF1B neutralizing antibody. Briefly, MC3T3-E1 cells were pre-treated with the neutralizing antibody at the concentration of 0.2  $\mu$ g/ml for 1h, and were then cultured in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$ . Cells treated with 0.2  $\mu$ g/ml of the normal rabbit IgG negative control antibody served as negative controls. Seven days or 14 days after the treatment, we found that the blockage of the binding between TNF- $\alpha$  and TNFR2 significantly down-regulated the ALP activity enhanced by TNF- $\alpha$  (Fig. 4a). Similarly, 24h or 48h after the treatment, the positive effect of the TNF- $\alpha$  treatment on expressions of RUNX2 and BSP was partly reversed by the blockage of the TNF- $\alpha$ -

TNFR2 binding. However, the positive effect of TNF- $\alpha$  on the expression of EphB4 showed no changes upon the treatment of the neutralizing antibody against TNFR2 (Fig. 4b-e).

To explore the role of EphB4 in TNF- $\alpha$ -enhanced osteogenic differentiation and TNFR2 expression, EphB4 forward signaling was suppressed by a potent inhibitor of EphB4 auto-phosphorylation, NVP-BHG712. Briefly, MC3T3-E1 cells were seeded in 6-well plates at a density of  $1.2 \times 10^5$  cells per well and were pretreated with 200 nM NVP-BHG712 in the regular culture medium for 1h. Cells were then incubated in osteogenic induction medium supplemented with 200 nM NVP-BHG712 and/or 0.5 ng/ml TNF- $\alpha$ . MC3T3-E1 cells cultured in osteogenic induction medium served as controls. Seven days or 14 days after the treatment, we found that NVP-BHG712 treatment significantly down-regulated TNF- $\alpha$ -stimulated ALP activity in MC3T3-E1 cells (Fig. 5a). Twenty-four hours or 48 hours after the treatment, changes in mRNA and protein expression levels of RUNX2 and BSP demonstrated a similar pattern to that observed in the ALP activity. Interestingly, 0.5ng/ml TNF- $\alpha$ -stimulated TNFR2 expression was also partly reversed when EphB4 was inhibited by NVP-BHG712 treatment (Figs. 5b-e), [signifying](#) that enhanced EphB4 signaling can modulate TNFR2 expression in TNF- $\alpha$  activated osteogenic process.

### **EphB4/TNFR2/ ERK/MAPK signaling pathway comprises a signaling axis to mediate the positive effect of TNF- $\alpha$ on osteogenic differentiation**

It was previously reported that MAPK signaling pathways play an important role in the osteogenic differentiation [26,27,28]. To investigate whether MAPK members were also involved in TNFR2 mediated pro-osteogenic differentiation under TNF- $\alpha$  stimulation, MC3T3-E1 cells were first treated with 0.5ng/ml TNF- $\alpha$  for 0, 5, 15, 30 and 60min, and levels of p38, *p*-p38, ERK1/2, *p*-ERK1/2, JNK1+2+3 and *p*-JNK1+2+3 were monitored using western blot analysis. As shown in Fig.6a, levels of *p*-p38, *p*-ERK1/2 and *p*-JNK1+2+3 were all significantly increased after TNF- $\alpha$  stimulation, with the most prominent increase observed at 15min after TNF- $\alpha$  stimulation. Then the pHLV-TNFR2siRNA1 cells and the pHLV-NC cells were cultured with TNF- $\alpha$  in regular culture medium for 15min, and found that the *p*-ERK1/2 level was significantly decreased in the pHLV-TNFR2siRNA1 cells compared with in the pHLV-NC cells (Fig. 6b). After MC3T3-E1 cells were pretreated with or without 200 nM NVP-BHG712 in the regular culture medium for 1h, 0.5ng/ml TNF- $\alpha$  was added into the medium and the cells were incubated for another 15min. Western blot analysis demonstrated that after the EphB4 forward signaling was inhibited, level of *p*-ERK1/2 was significantly down-regulated (Fig. 6c).

To further evaluate the role of ERK signaling in pro-osteogenic differentiation of TNF- $\alpha$ . MC3T3-E1 cells were cultured in the regular culture medium and pretreated with the ERK inhibitor U0126 (10 $\mu$ M). One hour after the pretreatment, the culture medium was switched to the osteogenic induction medium supplemented with TNF- $\alpha$  and/or the corresponding ERK inhibitors. Cells treated without the ERK inhibitors were served as controls. Seven days or 14 days after the treatment, we found that U0126 treatment significantly down-regulated the ALP activity enhanced by TNF- $\alpha$  (Fig. 6d). Three days later, RT-PCR and western blot analysis were performed to determine the expression levels of BSP and RUNX2. We found that the mRNA and protein expression of RUNX2 and BSP significantly decreased when compared with cells treated with TNF- $\alpha$  only (Fig. 6e, f). These results indicate that EphB4, TNFR2 and the ERK/MAPK

signaling pathways comprise a signaling axis to mediate the positive effect of TNF- $\alpha$  on osteogenic differentiation.

## Discussion

Under physiological conditions, TNF- $\alpha$  exists as a trimer, and binds to either TNFR1 (also known as TNFRSF1A or p55) or TNFR2 (also known as TNFRSF1B or p75) to exert its biological activity. TNFR1 is constitutively expressed on most cell surfaces, whereas TNFR2 gene expression is subject to both transcriptional and posttranscriptional regulation induced by external stimuli [29]. In addition, although the signaling pathways downstream of TNFR1 partially overlap with those of TNFR2, these two receptors often play different and independent roles in a variety of biological activities. For example, TNFR1 mediates cytotoxic effects as well as pro-inflammatory and pro-apoptotic effects, whereas TNFR2 is involved in maintaining the stabilization of Tregs and immunosuppressive facet [30,31,32]. TNFR1 is also reported to aggravate neuronal tissue destruction in a murine model of retinal ischemia, whereas TNFR2 is found to be effective in promoting neuroprotection [33]. Furthermore, various lines of evidence shows that PGRN-mediated anabolism of chondrocytes and bone formation depend on TNFR2 [34,35]. Moreover, TNFR2 activation plays a protective role in osteogenic differentiation and bone regeneration [3,4]. TNFR2 has been reported to play an important role in inflammation control, immune modulation, and neuronal protection. In this study, we investigated the role of TNFR2 in the pro-osteogenic effect of the low concentrations of TNF- $\alpha$ . Consistent with the previous findings showing that TNF treatment preferentially up-regulates TNFR2 on Tregs [36], we found that the TNFR2 expression was significantly enhanced at both mRNA and protein levels in murine pre-osteoblastic cells which were stimulated with TNF- $\alpha$ . Furthermore, lentivirus-mediated shRNA interference of TNFR2 as well as the blockage of the binding between TNF- $\alpha$  and TNFR2 both inhibited the pro-osteogenic effect of TNF- $\alpha$ , strongly indicating that TNFR2 has a pivotal effect on TNF- $\alpha$ -enhanced osteogenic differentiation.

Playing roles in a broad spectrum of biological processes, the ephrinB2-EphB4 bidirectional signaling system has also been reported to substantially contribute to bone remodeling, with the forward signaling through EphB4 into osteoblasts significantly enhancing osteogenic differentiation [22-25]. Our previous study strongly indicated that low concentrations of TNF- $\alpha$  activate EphB4 forward signaling to exert its pro-osteogenic effect in osteoblast precursor cells [16]. Given that both TNFR2 and EphB4 are osteogenic differentiation-associated membrane receptors and their expressions can be promoted by low concentrations of TNF- $\alpha$ , we investigated the interactions between EphB4 and TNFR2 in MC3T3-E1 pre-osteoblastic cells after stimulation with a low concentration of TNF- $\alpha$  in this study. We found that neither lentivirus-mediated shRNA interference of TNFR2 nor the blockage of the binding between TNF- $\alpha$  and TNFR2 showed effects on TNF- $\alpha$ -enhanced EphB4 expression. In contrast, the inhibited EphB4 forward signaling not only inhibited TNF- $\alpha$ -promoted osteogenic differentiation but also lowered TNF- $\alpha$ -stimulated TNFR2 expression, indicating that an activated EphB4 forward signaling is pivotal to TNF- $\alpha$ -enhanced TNFR2 expression and osteogenic differentiation.

It has been reported that TNF- $\alpha$  activates MAPK signaling pathways including p38, ERK and JNK [37]. In hepatic stellate cells, ephrinB2-EphB4 signaling stimulates ERK-dependent VEGF production and promotes chemotaxis [22]. In the study of ephrinB2-EphB4 signaling between osteoclasts and osteoblasts, ERK was found to be one of the downstream pathways of the EphB4 forward signaling in osteoblasts [23]. In addition, TNF- $\alpha$  can activate MAPK signaling pathways including p38, ERK and JNK pathways [37,38], among which ERK and JNK pathways have been proved to be important pathways for promoting osteogenic differentiation [39,40,41]. MAPK signaling pathways play important roles in the osteogenic differentiation [26,27,28]. Consistent with these findings, we found that p-p38, p-ERK1/2 and p-JNK1+2+3 were all significantly increased in MC3T3-E1 cells stimulated with TNF- $\alpha$ . As indicated by the western blot analysis, disruption of the TNFR2 signaling inhibited the ERK signaling pathway, while showed no inhibitory effect on the p38 and JNK signaling pathways, indicating that the TNFR2 signaling acts upstream of the ERK signaling pathway to promote osteogenic differentiation.

Besides EphB4/TNFR2, other signalings are also involved in TNF- $\alpha$  induced osteogenic differentiation. For example, A2BAR stimulation induces a significant enhancement of osteogenic processes, while TNF- $\alpha$  has been shown to modulate expression and functionality of A2BAR [17,18]. In addition, as shown in the present and other study, TNF- $\alpha$  can activate p38 signaling, whereas activated p38 /MAPK signaling pathway regulates the expression of BMP-2 in osteoblasts to direct the osteoblastic differentiation of mesenchymal stem cells [14]. Moreover, TNF- $\alpha$  was found to activate the Wnt/ $\beta$ -catenin pathway [42], while crosstalk between Wnt/ $\beta$ -Catenin and estrogen receptor signaling synergistically promotes osteogenic differentiation of mesenchymal progenitor cells [43]. However, what is the relation between the ephB4/TNFR2/ERK/MAPK pathways and these different pathways in TNF- $\alpha$  induced osteogenic differentiation remains to be further explored.

## Conclusions

Our findings strongly indicate that a low concentration of TNF- $\alpha$  enhances osteogenic differentiation *via* the activation of a series of signaling events. Briefly, the low concentration of TNF- $\alpha$  firstly stimulates EphB4 expression and enhances EphB4 forward signaling inside the osteoblastic cells, which results in the up-regulation of TNFR2. As a result, activation of ERK is initiated, which eventually enhances the expressions of bone marker genes and promotes osteogenic differentiation. In summary, EphB4, TNFR2 and the ERK/MAPK signaling pathways comprise a signaling axis to mediate the positive effect of TNF- $\alpha$  on osteogenic differentiation.

## Methods

### Cell culture and reagents

MC3T3-E1 mouse pre-osteoblast cell line, obtained from Shandong Provincial Key Laboratory of Oral Tissue Regeneration, were maintained with 5% CO<sub>2</sub> at 37°C in  $\alpha$ -MEM (Hyclone, Logan, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, USA), 100 U/ml penicillin (Solarbo, Beijing, China) and 100  $\mu$ g/ml streptomycin (Solarbo, Beijing, China). For osteogenic induction, the cells were cultured in

$\alpha$ -MEM supplemented with 5% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin,  $10^{-8}$  mol/l dexamethason (Sigma, St, Louis, MO), 50 mg/l ascorbic acid (Sigma, St, Louis, MO) and 10 mmol/l  $\beta$ -glycerophosphate (Sigma, St, Louis, MO). The medium was switched every two days.

TNF- $\alpha$  was purchased from PeproTech (Rocky Hill, NJ, USA) and the concentration of 0.5 ng/ml was used based on our preliminary concentration screening. The neutralizing antibody against TNFR2, anti-mouse TNFR2/CD120b/TNFRSF1B neutralizing antibody (catalog# 50128-RN204), was from Sino Biological (Beijing, China). A normal rabbit IgG, which is an unconjugated rabbit polyclonal antibody and is not directed against any known antigen, was purchased from Cell Signaling Technology (catalog# 2729, Beverly, MA, USA) and used as a negative control. The inhibitor of EphB4 auto-phosphorylation, NVP-BHG712, was obtained from MedChemExpress (HY-13258). The ERK inhibitor U0126 were purchased from Cell Signaling Technology (Danvers, MA, USA).

### Cell proliferation assay

Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to evaluate cell proliferation/survival. Briefly, MC3T3-E1 cells were seeded in 96-well plates at a density of  $3 \times 10^3$  cells per well and cultured in regular culture medium for 24h. The medium was then switched to  $\alpha$ -MEM supplemented with 0.1% FBS and 0.5ng/ml murine TNF- $\alpha$  (Peprotech Inc, Rocky Hill, NJ, USA). After 24h or 48h of TNF- $\alpha$  stimulation, 10  $\mu$ l of CCK-8 solution was added to each well and the plates were incubated for 2.5h at 37°C. The optical density was measured at 450 nm using the SPECTROstar Nano Microplate Reader (BMG Labtech Inc, Ortenberg, Germany).

### RNA isolation & reverse transcription-polymerase chain reaction (RT-PCR)

Our previous study has proved that low doses of TNF- $\alpha$  promotes expression of osteogenic differentiation associated markers at 7 and 14 days and mineralization at 21 days 16. Main aim of this study is to explore whether there exists the crosstalk between TNFR2 and EphB4 signaling and, if so, what is downstream signaling pathway in the pro-osteogenic effect of low concentrations of TNF- $\alpha$ , thus, MC3T3-E1 cells with or without gene modified were cultured for 24h or 48h rather than longer time period to observe synchronous change of osteogenic markers and signaling molecules. For mRNA assays, total RNA was extracted from the cells using Trizol<sup>®</sup> reagent (TaKaRa Biotech, Tokyo, Japan) according to the manufacturer's instructions, and was reverse transcribed using Reverse Transcriptase (TaKaRa Biotech, Tokyo, Japan). RT-PCR was performed using SYBR<sup>®</sup> Primix Ex Taq<sup>™</sup> (TaKaRa Biotech, Tokyo, Japan) with Roche 480 LightCycler System (Roche Diagnostics, Mannheim, Germany). Each sample was prepared in triplicate, and each experiment was repeated at least 3 times. GAPDH was used as a loading control. The sequences of the primers for amplification of mouse *Tnfr2*, *Runx2*, *Bsp*, *Ephb4* and *Gapdh* were shown in Table 1. The relative gene expression levels were calculated using the  $2^{-\Delta\Delta CT}$  method.

### Western blot analysis

Total cell lysates were extracted from MC3T3-E1 cells by incubation with ice-cold RIPA (Solarbo, Beijing, China) containing 1% PMSF (Solarbo, Beijing, China) for 30min, and the protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Solarbo, Beijing, China). For immunoblot analysis, 20 µg of protein lysates per sample were denatured in 5×SDS-PAGE loading buffer (Beyotime, Shanghai, China) at 100°C for 5min. The samples were then run on 10% SDS-PAGE gels (Beyotime, Shanghai, China), and transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA) for 1h at 100 V. The membranes were subsequently blocked with 5% defatted milk for 1h at room temperature and incubated with the primary antibodies overnight at 4°C. The anti-mouse primary antibodies used in this study were listed as following: RUNX2 (1:1000, catalog no. 12556S; CST, Danvers, MA, USA), BSP (1:1000, catalog no. 5468S CST, Danvers, MA, USA), EphB4 (1:1000, catalog no. A00690; Boster, China), TNFR2 (1:1000, catalog no. ab19139; abcam, Danvers, MA, USA), p38 (1:1000, catalog no. ab170099; abcam, Danvers, MA, USA), p-p38 (1:1000, catalog no. ab195049; abcam, Danvers, MA, USA), JNK1+2+3 (1:1000, catalog no. ab179461; abcam, Danvers, MA, USA), p-JNK1+2+3 (1:5000, catalog no. ab124956; abcam, Danvers, MA, USA), ERK1/2 (1:10000, catalog no. ab184699; abcam, Danvers, MA, USA), and p-ERK1/2 (1:8000, catalog no. ab76299; abcam, Danvers, MA, USA). The membranes were then incubated with an HRP-linked goat anti-rabbit secondary antibody (1:5000, catalog no. 7074P2; CST, Danvers, MA, USA) for 1h at room temperature. For normalization, defatted milk-blocked membranes were incubated with an HRP-linked anti-mouse GAPDH primary antibody (1:20000, catalog no. HRP-60004; Proteintech, Wuhan, China) for 1h at room temperature. Protein bands were visualized using the Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA). Quantification of the band intensity was carried out using the Image J Software (NIH, Bethesda, MD, USA).

### **ALP activity assay**

After osteogenic induction for 7d or 14d, the cell lysates were extracted from the MC3T3-E1 cells using 1% Triton X-100 for 30min on ice. The cell lysates were centrifuged at  $1.2 \times 10^4$  g for 5min at 4°C, and the ALP activity was evaluated using an Alkaline Phosphatase Assay Kit according to the instructions of the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). ALP activity was calculated according to the concentration of the phenol in a standard well and adjusted according to the protein concentration of each sample.

### **Immunofluorescent Staining**

For immunofluorescent staining, MC3T3-E1 cells were washed with cold PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in 1% bovine serum albumin for 30min, blocked with 5% bovine serum albumin for 1 hour at room temperature, and incubated overnight at 4°C with polyclonal rabbit anti-TNFR2 antibody (1:100 dilution, 19272-1-AP, Proteintech, USA). Cells were then incubated with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (1:100 dilution, SA00006-4, Proteintech, USA). The cell nuclei were stained with 4, 6-diamino-2-phenylindole (DAPI). Stained MC3T3-E1 cells were visualized under a fluorescent microscope (Olympus, BX51, Japan), and images were captured by a CCD camera (CoolSNAP-Pro cf., Media Cybernetics, USA). The fluorescence intensity was counted in selected merged microscopic images by Image-Pro Plus 6.0 software.

## Lentivirus-mediated shRNA interference targeting TNFR2

siRNAs specifically targeting TNFR2 were designed and synthesized by Hanbio Biotechnology Co., Ltd. (Shanghai, China). An siRNA with no homology to any known mouse or human gene was also synthesized to serve as a negative control. Synthesized siRNAs were duplexed, ligated into the pHBLV-ZsGreen-PURO expression vector (Hanbio Biotechnology Co., Ltd., Shanghai, China), and confirmed by gene sequencing. The resulting lentiviral vectors were then transfected into human 293T cells with pSPAX2 and pMD2G using Lipofiter™ Liposomal Transfection Reagent (Hanbio Biotechnology Co., Ltd., Shanghai, China). Forty-eight hours and 72 hours after the transfection, the supernatant was collected twice, and centrifuged at 2,000g for 10min to remove cell debris. The lentiviral particles were then concentrated by centrifugation at 82,700g for 2 hours and resuspended in opti-MEM. After the titration, the lentiviral particles were stored at -80°C.

For lentiviral infection, MC3T3-E1 cells were seeded onto six-well plates and cultured in the regular culture medium for 12h. Cells were then incubated with the corresponding lentiviral particles (MOI=50) in 1 ml of the regular culture medium supplemented with 5µg/ml polybrene. Four hours later, another 1 ml of the regular culture medium containing 5µg/ml polybrene was added to each well. The plates were incubated at 37°C for 16h before the medium was switched to the regular culture medium. Forty-eight hours after the lentiviral transduction, green fluorescence (from ZsGreen) was captured by a fluorescence microscope (Olympus IX81, Tokyo, Japan). Stably transduced cells were selected with 8µg/ml puromycin (Sigma, St. Louis, MO) and were maintained in culture medium containing 2µg/ml puromycin.

## Statistical analysis

All data were expressed as the means ± standard error of the mean (SEM) from at least three replicates for each experiment. Differences between more than two experimental groups and negative control group were analyzed by one-way ANOVA followed by Tukey HSD comparison test. Variance between two groups was compared by two-way t-test with GraphPad Prism software (version 6, by MacKiev Software, Boston, MA, USA).  $p < 0.05$  was considered statistically significant.

## Availability of data and materials

Not applicable.

## List Of Abbreviations

**TNF-α:** tumor necrosis factor alpha

**TNFR1:** tumor necrosis factor receptor 1

**TNFR2:** tumor necrosis factor receptor 2

**PGRN:** progranulin

**MSCs:** mesenchymal stem cells

**A2BARs:** A2B receptors

**FBS:** fetal bovine serum

**RT-PCR:** RNA isolation & reverse transcription-polymerase chain reaction

**BCA:** bicinchoninic acid

**PVDF:** polyvinylidene fluoride

**ALP:** Alkaline phosphatase

## **Declarations**

### **Ethics approval and consent to participate**

This article does not contain any studies with human participants or animals performed by any of the authors.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

All data generated or analysed during this study are included in this published article.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

YZ, JZ and PSY conceived and designed the experiments, managed the preparation of the manuscript; YZ and CZY performed the experiments; SHG and LMW analyzed the data; YZ wrote the manuscript. All authors read and approved the final manuscript.

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## Tables

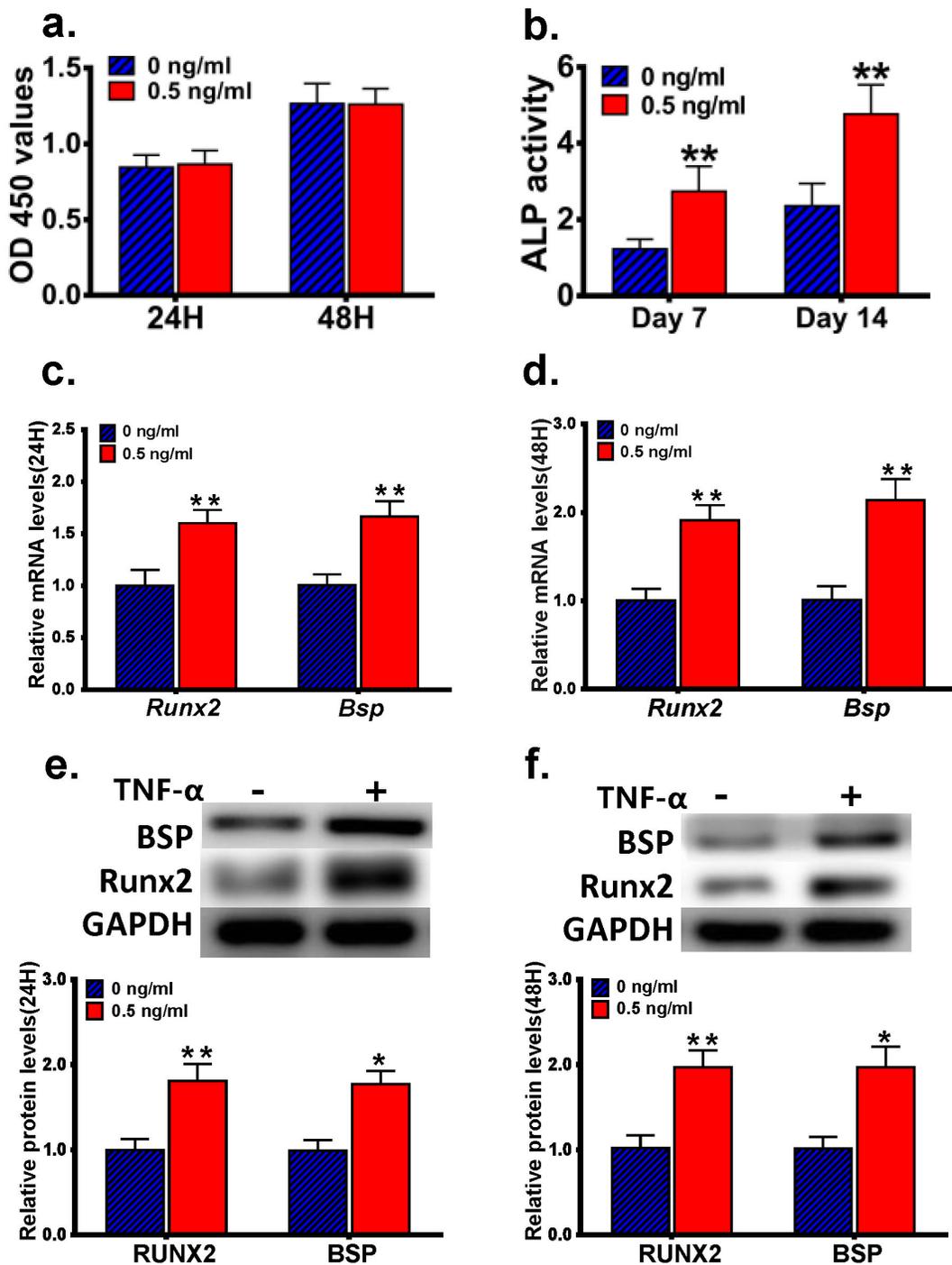
**Table 1.** Sequences of the synthesized siRNAs and shRNAs specifically targeting murine TNFR2

<b>Control siRNA</b>	TTCTCCGAACGTGTCACGTAA	
<b>Control shRNA</b>	<b>Top</b>	GATCCGTTCTCCGAACGTGTCACGTAATTCAAGAGATTACGTGACACGTTCCGAGAATTTTTTC
	<b>Bottom</b>	AATTGAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGAATTACGTGACACGTTCCGAGAACG
<b>siRNA1</b>	GGGACGTTCTCTGACACCACATCAT	
<b>shRNA1</b>	<b>Top</b>	GATCCGGGACGTTCTCTGACACCACATCATTCAAGAGAATGATGTGGTGTGACAGAGAACGTCCCTTTTTTG
	<b>Bottom</b>	AATTCAAAAAAGGGACGTTCTCTGACACCACATCATTCTCTTGAATGATGTGGTGTGACAGAGAACGTCCCG
<b>siRNA2</b>	CCACAGTTCTCAGTGCTCTTCCCAA	
<b>shRNA2</b>	<b>Top</b>	GATCCGCCACAGTTCTCAGTGCTCTTCCCAATTCAAGAGATTGGGAAGAGCACTGAGAACTGTGGTTTTTG
	<b>Bottom</b>	AATTCAAAAAACCACAGTTCTCAGTGCTCTTCCCAATCTCTTGAATTGGGAAGAGCACTGAGAACTGTGGCG
<b>siRNA3</b>	CGTGTGAGACTACAGAGACACTGCA	
<b>shRNA3</b>	<b>Top</b>	GATCCGCGTGTGAGACTACAGAGACACTGCATTCAAGAGATGCAGTGTCTCTGTAGTCTCACACGTTTTTG
	<b>Bottom</b>	AATTCAAAAAACGTGTGAGACTACAGAGACACTGCATCTCTTGAATGCAGTGTCTCTGTAGTCTCACACGCG

**Table 2.** Primer sequences for qRT-PCR

<b>Gene</b>	<b>Forward (5'~3')</b>	<b>Reverse (5'~3')</b>
<i>Runx2</i>	CCCAGCCACCTTTACCTACA	TATGGAGTGCTGCTGGTCTG
<i>Bsp</i>	CAGGGAGGCAGTGACTCTTC	AGTGTGGAAAGTGTGGCGTT
<i>Ephb4</i>	GCGGAAAGCAACAAAGTA	CGGCAGCGTACAGCATAAGT
<i>Tnfr2</i>	CTGCGCCTTGAAAACCCATT	GATGCTACAGATGCGGTGGG
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

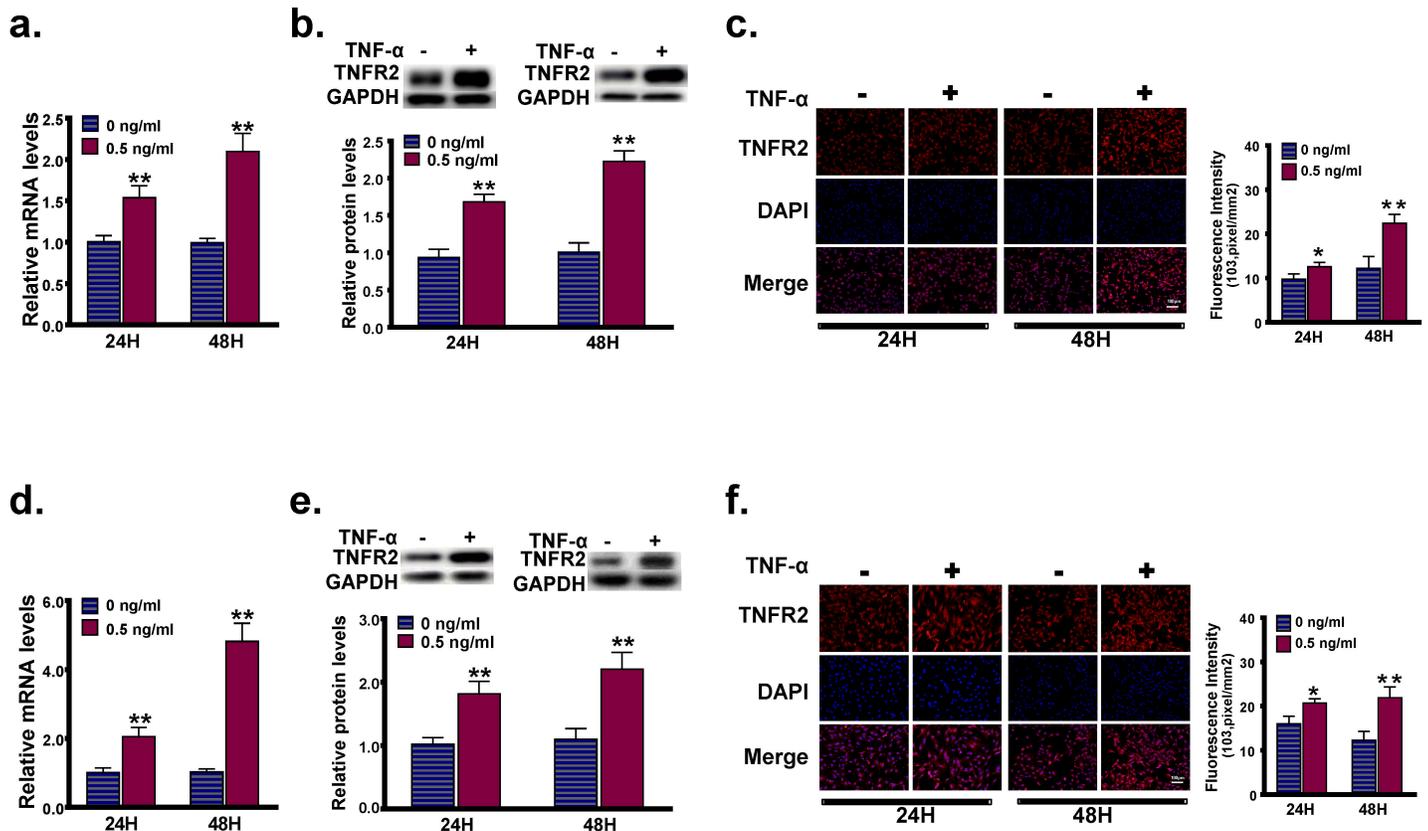
## Figures



**Figure 1**

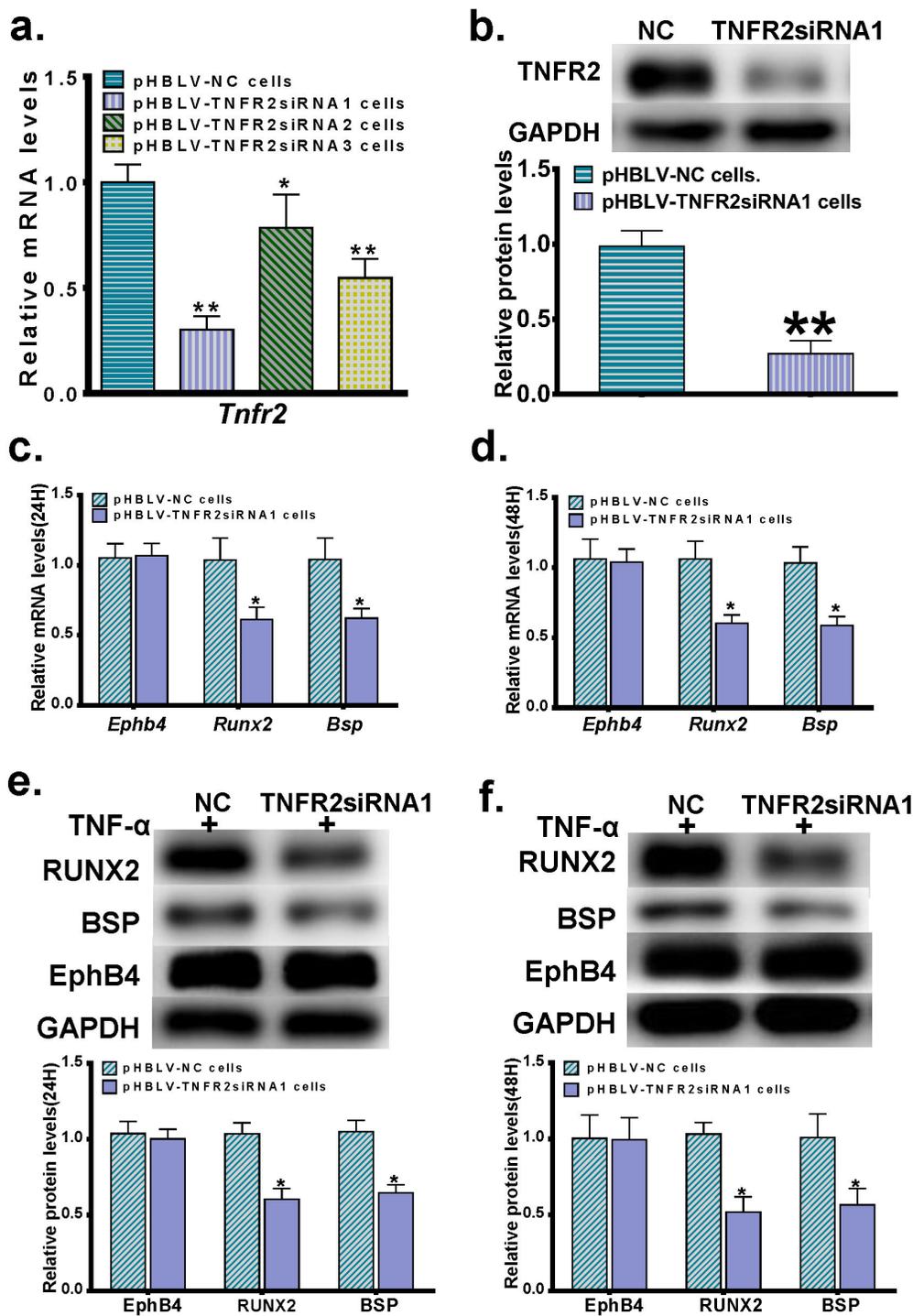
The effect of a low concentration of TNF- $\alpha$  on the proliferation/survival and osteogenic differentiation of MC3T3-E1 cells. (a) MC3T3-E1 cells were treated with or without 0.5ng/ml TNF- $\alpha$  for 24h or 48h, and subjected to CCK-8 assay. No statistically significant difference in cell proliferation/survival was detected in MC3T3-E1 cells treated with 0.5ng/ml TNF- $\alpha$  when compared with the control cells. (b) MC3T3-E1 cells were cultured in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$  for 7d or

14d, and the ALP activity in these cells were determined. (c, d) MC3T3-E1 cells were cultured in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$  for 24h (c) or 48h (d), and the mRNA levels of Runx2 and Bsp were determined using RT-PCR. (e, f) MC3T3-E1 cells were cultured in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$  for 24h (e) or 48h (f), and the protein levels of RUNX2 and BSP were determined using western blot analysis. \*,  $p < 0.05$  vs. the 0ng/ml group; \*\*,  $p < 0.01$  vs. the 0ng/ml group.



**Figure 2**

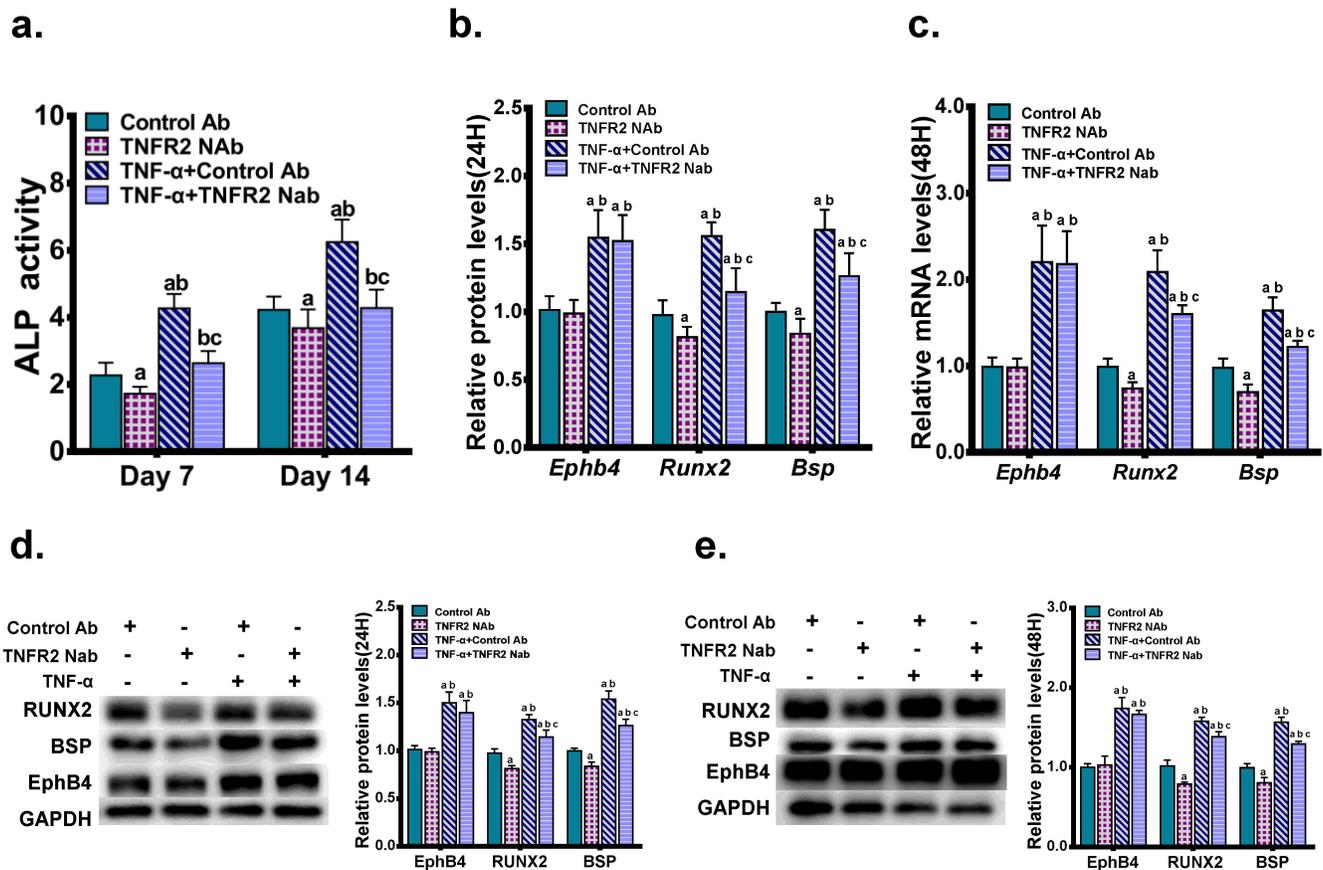
The effect of a low concentration of TNF- $\alpha$  on the expression of TNFR2 in MC3T3-E1 cells. (a-c) MC3T3-E1 cells were treated with or without 0.5ng/ml TNF- $\alpha$  in the regular culture medium for 24h or 48h, and the Tnfr2 expression level was determined using RT-PCR (a), western blot (b) and immunofluorescence staining for TNFR2 (c). (d-f) MC3T3-E1 cells were cultured in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$  for 24h or 48h, and the Tnfr2 expression level was determined using RT-PCR (d), western blot (e) and immunofluorescence staining for TNFR2 (f). \*,  $p < 0.05$  vs. the 0ng/ml group; \*\*,  $p < 0.01$  vs. the 0ng/ml group.



**Figure 3**

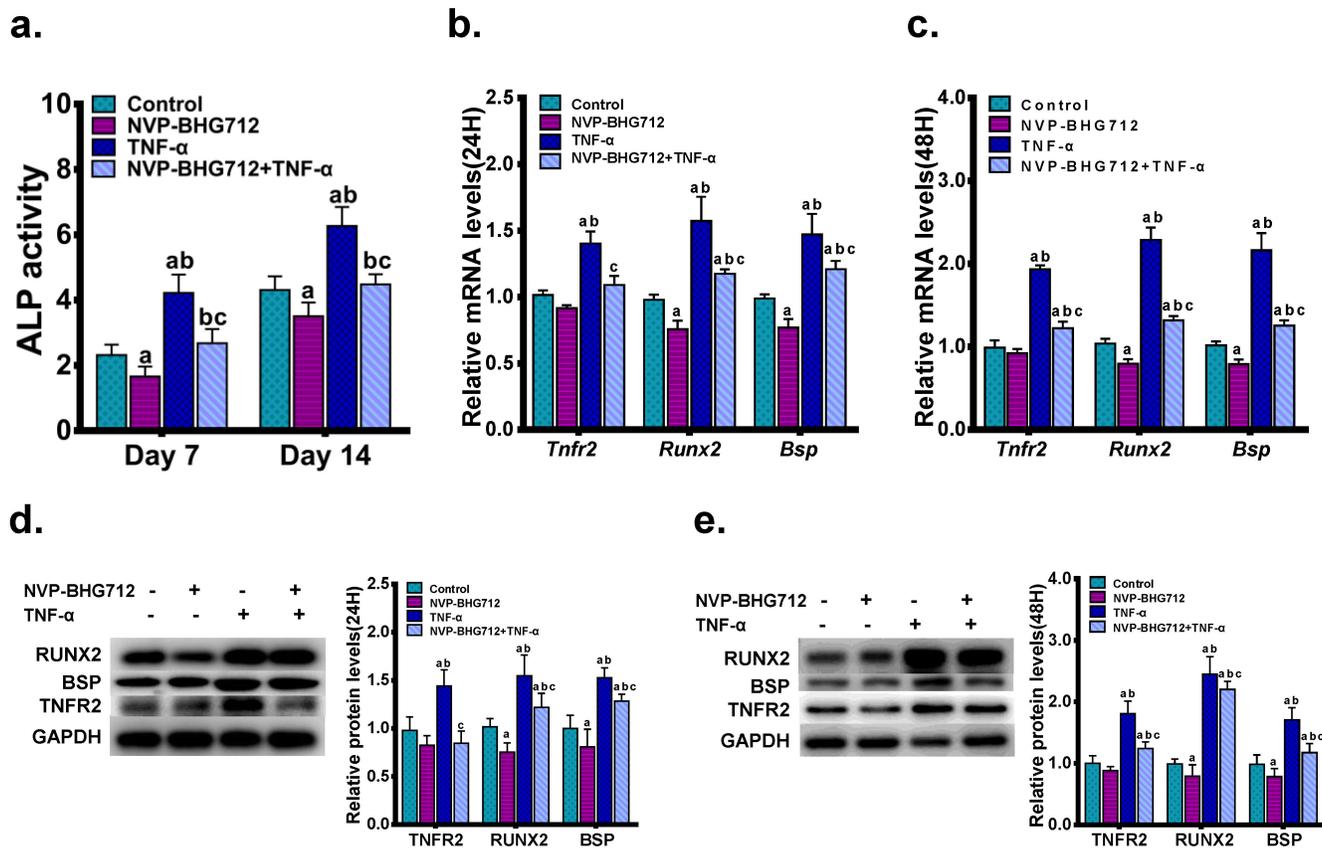
The effect of the lentivirus-mediated shRNA interference of TNFR2 on TNF- $\alpha$ -stimulated EphB4 expression and osteogenic differentiation. (a) MC3T3-E1 cells stably transduced with lentiviral particles were selected with puromycin and named as pHBLV-TNFR2siRNA1 cells, pHBLV-TNFR2siRNA2 cells, pHBLV-TNFR2siRNA3 cells and pHBLV-NC cells, respectively. The mRNA levels of *Tnfr2* were determined in these cells, among which the pHBLV-TNFR2siRNA1 cells displayed the highest TNFR2 gene silencing efficiency

and were selected to continue the following studies. (b) TNFR2 protein levels in pHBLV-TNFR2siRNA1 cells and pHBLV-NC cells. (c, d) mRNA levels of Ephb4, Runx2 and Bsp in pHBLV-TNFR2siRNA1 cells and pHBLV-NC cells cultured in the osteogenic induction medium supplemented with 0.5ng/ml TNF- $\alpha$  for 24h (c) or 48h (d). (e, f) Protein levels of EphB4, RUNX2 and BSP in pHBLV-TNFR2siRNA1 cells and pHBLV-NC cells cultured in the osteogenic induction medium supplemented with 0.5ng/ml TNF- $\alpha$  for 24h (e) or 48h (f). \*,  $p < 0.05$  vs. the pHBLV-NC group; \*\*,  $p < 0.01$  vs. the pHBLV-NC group.



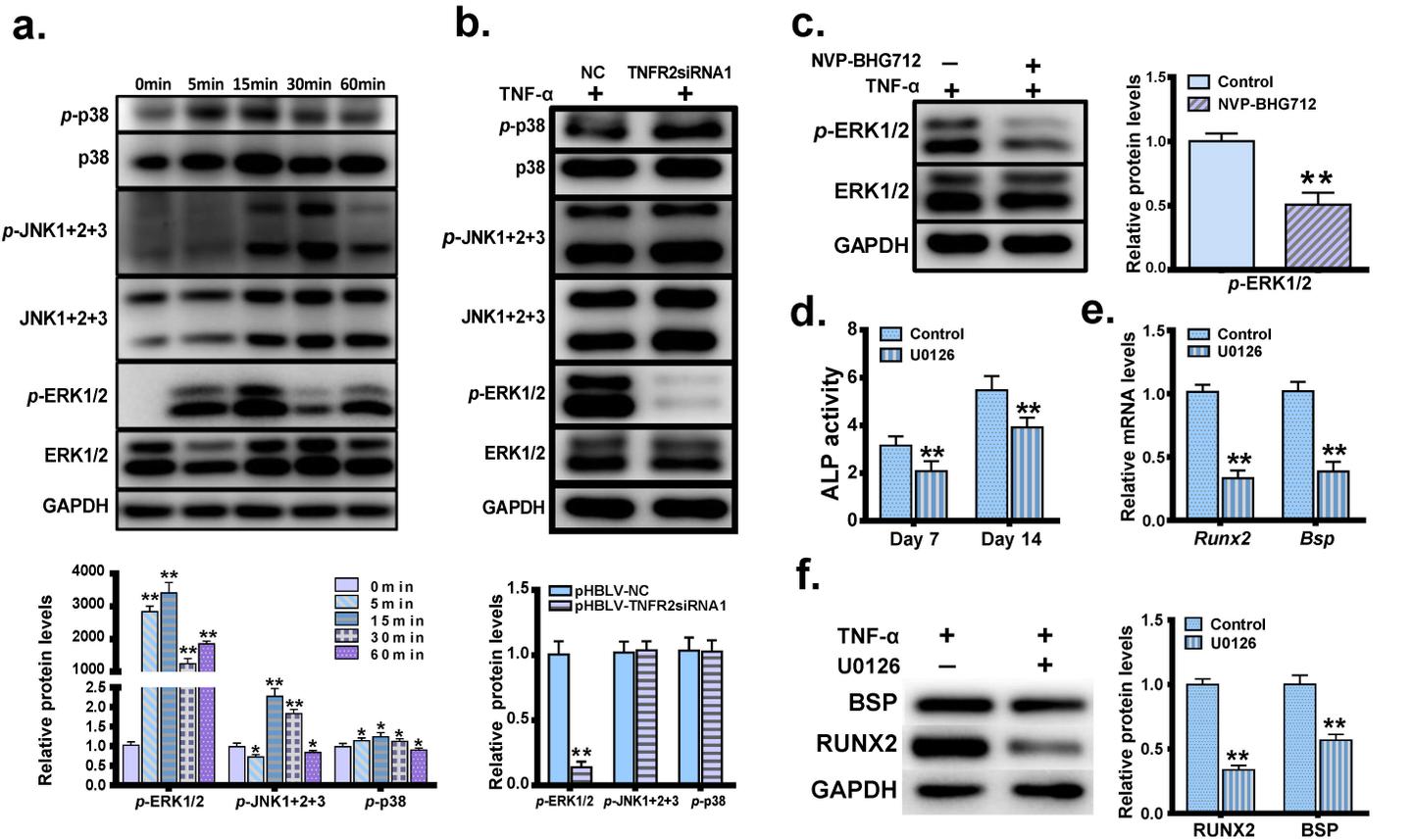
**Figure 4**

The effect of the impaired binding between TNF- $\alpha$  and TNFR2 on TNF- $\alpha$ -stimulated EphB4 expression and osteogenic differentiation. MC3T3-E1 cells were treated with an anti-mouse TNFR2/ CD120b/TNFRSF1B neutralizing antibody (TNFR2 NAb) at the concentration of 0.2  $\mu\text{g/ml}$ , and were cultured in the osteogenic induction medium supplemented with or without 0.5ng/ml TNF- $\alpha$ . Cells treated with 0.2  $\mu\text{g/ml}$  of the normal rabbit IgG negative control antibody (control Ab) served as negative controls. (a) ALP activities were determined 7d or 14d after the treatment. (b, c) mRNA levels of Ephb4, Runx2 and Bsp were determined after 24h (b) or 48h (c). (d, e) Protein levels of EphB4, RUNX2 and BSP were determined after 24h (d) or 48h (e). a,  $p < 0.05$  vs. the control Ab group; b,  $p < 0.05$  vs. the TNFR2 NAb group; c,  $p < 0.05$  vs. the TNF- $\alpha$ +control Ab group.



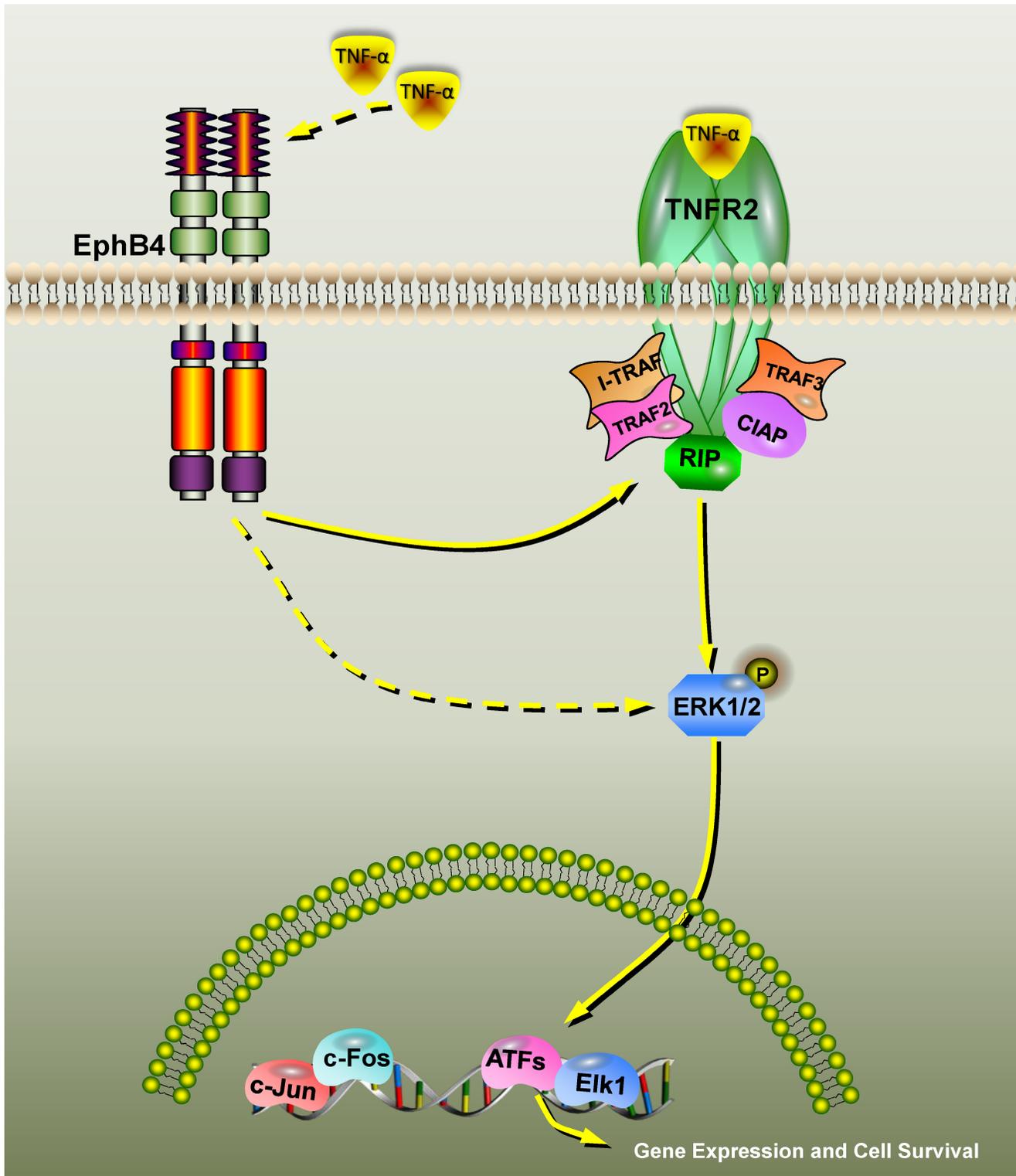
**Figure 5**

The effect of inhibited EphB4 forward signaling on TNF- $\alpha$ -stimulated TNFR2 expression and osteogenic differentiation. (a) A potent inhibitor of EphB4 auto-phosphorylation, NVP-BHG712, was used to suppress EphB4 forward signaling. MC3T3-E1 cells were pretreated with 200 nM NVP-BHG712 in the regular culture medium for 1h. Cells were then incubated in osteogenic induction medium supplemented with 200 nM NVP-BHG712 and/or 0.5ng/ml TNF- $\alpha$  for 7d or 14d. MC3T3-E1 cells cultured in osteogenic induction medium served as controls. The ALP activities were determined. (b, c) MC3T3-E1 cells were pretreated with 200 nM NVP-BHG712 for 1h in the regular culture medium, and then incubated in osteogenic induction medium supplemented with 200 nM NVP-BHG712 and/or 0.5ng/ml TNF- $\alpha$ . Cells cultured in osteogenic induction medium served as controls. mRNA levels of *Tnfr2*, *Runx2* and *Bsp* were determined after 24h (b) or 48h (c) of incubation. (d, e) MC3T3-E1 cells were pretreated with 200 nM NVP-BHG712 for 1h in the regular culture medium, and then incubated in osteogenic induction medium supplemented with 200 nM NVP-BHG712 and/or 0.5ng/ml TNF- $\alpha$ . Cells cultured in osteogenic induction medium served as controls. Protein levels of TNFR2, RUNX2 and BSP were determined after 24h (d) or 48h (e) of incubation. a, p<0.05 vs. the control group; b, p<0.05 vs. the NVP-BHG712 group; c, p<0.05 vs. the TNF- $\alpha$  group.



**Figure 6**

EphB4, TNFR2 and MAPK signaling pathways comprise a signaling axis to mediate the positive effect of TNF- $\alpha$  on osteogenic differentiation. (a) Levels of p38, p-p38, ERK1/2, p-ERK1/2, JNK1+2+3 and p-JNK1+2+3 in MC3T3-E1 cells treated with TNF- $\alpha$  for 0min, 5min, 15min, 30min and 60min. (b) Levels of p38, p-p38, ERK1/2, p-ERK1/2, JNK1+2+3 and p-JNK1+2+3 in the pHBLV-TNFR2siRNA1 cells and the pHBLV-NC cells treated with or without 0.5ng/ml TNF- $\alpha$  in regular culture medium for 15min. (c) MC3T3-E1 cells were pretreated with or without 200 nM NVP-BHG712 in the regular culture medium for 1h, 0.5ng/ml TNF- $\alpha$  was added into the medium and the cells were incubated for another 15min. Levels of ERK1/2, p-ERK1/2 were determined. MC3T3-E1 cells were cultured in the regular culture medium and pretreated with the ERK inhibitor U0126 (10 $\mu$ M). One hour after the pretreatment, the culture medium was switched to the osteogenic induction medium supplemented with 0.5ng/ml TNF- $\alpha$  and/or the corresponding ERK inhibitors. Cells treated without the ERK inhibitors were served as controls. (d) ALP activities were determined 7d or 14d after the treatment. (e, f) Three days after the incubation, mRNA levels of Runx2 and Bsp were determined (e), western blot analysis was performed to determine the expression levels of BSP and RUNX2(f). \*,  $p < 0.05$  vs. the control group; \*\*,  $p < 0.01$  vs. the control group.



**Figure 7**

Schematic diagram of the EphB4, TNFR2 and ERK/MAPK signaling pathways. A low concentration of TNF- $\alpha$  first enhances the expression of EphB4, then promoted the expression of TNFR2. The elevated TNFR2 level leads to the activation of the ERK signaling pathway, which eventually enhances the osteogenic differentiation of MC3T3-E1 cells. TNFR2, tumor necrosis factor receptor 2; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase.