Received: 29 August 2018 | Revised: 9 February 2019 | Accepted: 14 February 2019

DOI: 10.1002/jcp.28440

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

ELP2 negatively regulates osteoblastic differentiation impaired by tumor necrosis factor α in MC3T3–E1 cells through STAT3 activation

Chang-Peng Xu[1] | Hong-Tao Sun[1] | Ya-Jun Yang[2] | Zhuang Cui[3] | Jian Wang[4] | Bin Yu[3] | Fa-Zheng Wang[5] | Qing-Po Yang[5] | Yong Qi[1]

[1]Department of Orthopaedics, Guangdong Second Provincial General Hospital, Guangzhou, Guangdong, People's Republic of China

[2]Department of Pharmacology, Guangdong Medical University, Zhanjiang, Guangdong, People's Republic of China

[3]Department of Orthopaedics and Traumatology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, People's Republic of China

[4]Department of Orthopaedics, The Inner Mongolia People's Hospital, Hohhot, Inner Mongolia, People's Republic of China

[5]Department of Orthopaedics, The First People's Hospital of Kashgar Prefecture, Kashgar, Xinjiang, People's Republic of China

Correspondence
Chang-Peng Xu and Yong Qi, Department of Orthopaedics, Guangdong Second Provincial General Hospital, NO.466 Xingang Road, Haizhu, 510317 Guangzhou, People's Republic of China.
Email: gd2hxcp@163.com (C.-P.X.); 7256401@163.com (Y.Q.)

Funding information
National Natural Science Fundation for Youths of China, Grant/Award Number: 81501902; Guangdong Province Natural Science Foundation, Grant/Award Numbers: 2014A030310451, 2017A030313736; Science and Technology Planning Project of Guangzhou, Grant/Award Number: 201804010226; Science Foundation of Guangdong Second Provincial General Hospital, Grant/Award Number: YQ2016-003; National Natural Science Fundation of China, Grant/Award Number: 81560368; Science and Technology Assistance Project of Xinjiang Province, Grant/Award Number: 2018E02056; Natural Science Foundation of Xinjiang Province, Grant/Award Number: 2018D01C014

Abstract

Tumor necrosis factor-α (TNF-α) is a pluripotent signaling molecule. The biological effect of TNF-α includes slowing down osteogenic differentiation, which can lead to bone dysplasia in long-term inflammatory microenvironments. Signal transducer and activator of transcription 3 (STAT3)-interacting protein 1 (StIP1, also known as elongator complex protein 2, ELP2) play a role in inhibiting TNF-α-induced osteoblast differentiation. In the present study, we investigated whether and how ELP2 activation mediates the effects of TNF-α on osteoblastic differentiation. Using in vitro cell cultures of preosteoblastic MC3T3-E1 cells, we found that TNF-α inhibited osteoblastic differentiation accompanied by an increase in ELP2 expression and STAT3 activation. Forced ELP2 expression inhibited osteogenic differentiation of MC3T3-E1 cells, with a decrease in the expression of osteoblast marker genes, alkaline phosphatase activity, and matrix mineralization capacity. In contrast, ELP2 silencing ameliorated osteogenic differentiation in MC3T3-E1 cells, even after TNF-α stimulation. The TNF-α-induced inhibitory effect on osteoblastic differentiation was therefore mediated by ELP2, which was associated with Janus kinase 2 (JAK2)/STAT3 activation. These results suggest that ELP2 is upregulated at the differentiation of MC3T3-E1 cells into osteoblasts and inhibits osteogenic differentiation in response to TNF-α through STAT3 activation.

KEYWORDS
ELP2, osteoblastic differentiation, signal transducer and activator of transcription 3, tumor necrosis factor-α

1 | INTRODUCTION

Human bones constantly renew themselves with the metabolism of the body. The balance of bone mass is maintained by the osteogenic effects of osteoblasts and the bone resorption effects of osteoclasts (Almeida et al., 2017). However, a few of persistent inflammation, such as systemic lupus erythaematosus, rheumatoid arthritis, and traumatic fractures, is associated with bone formation and imposed an adverse influence on bone mass balance and bone regeneration (Mcbridegagyi, Mckenzie, Buettmann, Gardner, & Silva, 2015). Tumor necrosis factor-α (TNF-α) is a representative of the proinflammatory cytokines which is a catabolic factor for inflammatory responses in disease (Li et al., 2010). Persistent inflammation has a significant effect on bone regeneration. Once the signaling pathways are elucidated, possible strategies can be adopted to circumvent inflammation suppression of tissue regeneration (Tsialogiannis et al., 2009).

TNF-α inhibits osteoblast differentiation by downregulating the expression of key osteoblast markers such as Runx2 and decreasing the activity of alkaline phosphatase (ALP; Kaneki et al., 2006). Previous research of Yamazaki et al. (2009) and us have demonstrated that TNF-α inhibits osteoblast differentiation by activating the NF-κB pathway, directly leading to the elimination of Smad1 signaling (Li et al., 2010). Other reports also indicated that TNF-α-induced differentiation inhibition occurs via the SAPK/JNK signaling (Mukai et al., 2007). Although the inhibition effect of TNF-α has been reported, further studies are needed involving the detailed TNF-α signaling pathway. Our previous study on proteomics (Xu et al., 2015), used isobaric tags for relative and absolute quantitation (iTRAQ) combined with multiple reaction monitoring quantitative proteomics methods, revealed that ELP2 has a vital regulatory effect on TNF-α-induced osteoblast differentiation inhibition and ligand-dependent signal transducer and activator of transcription 3 (STAT3) activation in the presence of TNF-α.

The evolutionarily conserved elongator complex (EC) plays a role in gene regulation, is associated with various cellular activities, and has attracted cultural attention (Karlsborn et al., 2014). There are two subcomplexes in the whole EC structure, each composed of three subunits. The ELP123 (ELP1–ELP3) subunits are the core subcomplex, whereas the ELP456 (ELP4–ELP6) subunits have been suggested to be an ancillary subcomplex (Glatt, Séraphin, & Müller, 2012). ELP2, also known as STAT3-interacting protein 1 (StIP1), regulates the ligand-dependent activation of STAT3 (Collum, Brutsaert, Lee, & Schindler, 2000; Hawkes et al., 2002; S. Lu, Fan, Li, & Fan, 2018). STAT3 has been demonstrated to be able to be activated by TNF-α in many processes (Yoshimura, 2006). However, the above findings separately resulted from different cell sources and different experimental conditions in vitro, and the molecular mechanisms of ELP2 involved in the early stages of osteogenic differentiation in an inflammatory microenvironment are still poorly understood. The results of our quantitative proteomics and protein interaction analyses in our previous study led us to hypothesize that ELP2 may act downstream of TNF-α and negatively regulate osteoblast differentiation impaired by TNF-α through STAT3 activation (Xu et al., 2015).

MC3T3 is an osteoblast precursor cell line derived from *Mus musculus* (mouse) calvaria and it has been widely used as a model to investigate osteoblast differentiation in early stage (Son et al., 2008). Based on the background above, the aim of this study was to test the hypothesis that ELP2 promotes the deleterious effects of TNF-α on osteoblastic differentiation in mouse preosteoblastic MC3T3-E1 cells and that the underlying mechanism may involve ELP2 acting downstream of TNF-α while simultaneously promoting the activation of STAT3.

2 | MATERIALS AND METHODS

2.1 | Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and penicillin–streptomycin solution were purchased from Life Technologies Corporation (Carlsbad, CA). Dimethylsulfoxide, human recombinant TNF-α, and Alizarin Red Staining were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO).

2.2 | Cell culture and osteoblast differentiation

Preosteoblastic MC3T3-E1 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in α-modified Eagle's medium (α-DMEM; Thermo Fisher Scientific, Waltham, MA). A total of 10% (v:v) fetal bovine serum and 1% (v:v) each of penicillin, glutamine, streptomycin, and sodium pyruvate were added into the culture medium. Cells were cultured in six-well plates in 37°C under a humidified atmosphere of 5% CO_2. Osteogenic differentiation was induced by culture medium containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate, which were added at end of logarithmic phase. Then, cells were serum starved, followed by treatments of normal control vehicle, TNF-α at different concentrations as indicated.

2.3 | Plasmid constructs and cell transfection

The full-length nucleotide sequence of human ELP2 (NCBI: NM_021448.2) was amplified with real-time polymerase chain reaction (RT-PCR) with total RNA of MC3T3-E1 cells. The sequence was then cloned into the pcDNA3 expression vector (Invitrogen, San Diego, CA) to construct recombinant vector pcDNA3-ELP2 with the primers listed in Table 1. Then pcDNA3-ELP2 was transfected into MC3T3-E1 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and the pcDNA3 vectors without insertion were served as blank controls.

The ELP2 small interfering RNA (siRNA) and negative control siRNA were purchased from GenePharma (Shanghai, China). The sequences were designed as listed in Table 1. Cells in the exponential growth phase were subplated in 24-well plates at a density of 30–50% and cultured in the same condition for 24 hr. The cells were

TABLE 1 Primers/overexpression and RNA-interference sequences used in this study

Name	Sequence	
ELP2	5′-GCCATGAAGGACCTGTTTGT-3′(forward) 5′-GCCAGGCAGACAGAAAGAAC-3′(reverse)	For qPCR
Runx2	5′-GCTGTTAACTTCAAGTCCCT-3′(forward) 5′-GAAATCAAGTTCGAGGAAGC-3′(reverse)	
ALP	5′-CATGAGGGAGCGGTAGAG-3′(forward) 5′-TGGGTCCTATGATATCTCTGAT-3′(reverse)	
Osteonectin	5′-TTCCATCACGAAGAAGCC-3′(forward) 5′-AAACTACTGTTTCCCAAAGC-3′(reverse)	
Osterix	5′-AGTGGATACGTACAGAGGAT-3′(forward) 5′-AGGTATTTGACTTCAGACCC-3′(reverse)	
OPN	5′-GCTGAATTCTGAGGGACTAAC-3′(forward) 5′-CTGTAAAGCTTCTTCTCCTCTG-3′(reverse)	
OCN	5′-AGCACCAGAATCTATCTGAA-3′(forward) 5′-AATGCCTTGTTCTCCTCTTA-3′(reverse)	
β-actin	5′-AGATGTGGATCAGCAAGCA-3′ (forward) 5′-CCATGCCAATGTTGTCTCTT-3′ (reverse)	
pcDNA3-ELP2	5′-CTGGGATCCATGGTTTCTTCTGTGCTG-3′ (forward) 5′-CTGCTCGAGTCACAGTGCGCGTCTGTTAAC-3′ (reverse)	For overexpression
siRNA ELP2	5′-GACUGCCUAAUAAGAAUAUTT-3′ (forward) 5′-AUAUUCUUAUUAGGCAGUCTT-3′ (reverse)	For RNAi

Note. qRT-PCR: quantitative real-time polymerase chain reaction; siRNA: small interfering RNA.

then transfected with siRNA according to the manufacturer's instructions of Lipofectamine 2000.

2.4 | RNA extraction and quantitative PCR analysis

The total RNA samples of each cell group were extracted using Trizol reagent (Invitrogen) and the protocol was according to the manufacturer's instructions. The QuantiTect SYBR Green PCR Kit (Invitrogen) and the MX3005P multiplex quantitative PCR system (ABI PRISM, Waltham, MA) were used to quantify the messenger RNA (mRNAs) after the extraction procedure. β-actin gene was used as reference genes and three repeats were performed to keep accuracy. The primers used in this step are listed in Table 1. The relative expression levels of were calculated using the comparative $\Delta\Delta C_t$ method and fold changes were calculated by the equation $2^{-\Delta\Delta Ct}$. The fold-change values were further calculated and represented as the means ± standard error of mean of mRNA expression normalized to β-actin.

2.5 | Western blot analysis

After MC3T3-E1 cells treated with or without TNF-α, the expression levels of targeting proteins were determined using western blot assays. The procedures and antibodies were according to the previous report (Xu et al., 2015), that is anti-ELP2 (rabbit polyclonal to ELP2; ab18048; Abcam, Cambridge, UK), anti-p-Y705-STAT3 (BosterBio, Pleasanton, CA), anti-p-JAK2 (Tyr1007/1008; BosterBio), anti-STAT3 (BosterBio), anti-JAK2 (BosterBio) and anti-β-actin (BosterBio). The protein stripe were developed with horseradish peroxidase-conjugated secondary antibodies and ECL reagent (Pierce, Rockford, IL). The relative levels of individual proteins to control β-actin were analyzed by ImageJ2 software (LOCI, Madison, WI).

2.6 | Luciferase reporter assay

Luciferase reporter gene assay was performed to measure STAT3 transcriptional activity (Zhang, Xiao, Wang, Tian, & Zhang, 2011). In brief, MC3T3-E1 cells were transfected with control *Renilla* luciferase reporter plasmid blended with a firefly luciferase reporter plasmid containing the conserved STAT3 that bound site or with blank plasmid pGL6-TA-luc (Beyotime, Jiangsu, China) at a 1:10 ratio that used Lipofectamine 2000. After two days, the cells were treated with or without TNF-α (5 ng/ml) for 2 hr, and the luciferase activity in different groups of cells was determined by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase values were normalized against those of *Renilla* luciferase. All experiment was performed in three times.

2.7 | Immunofluorescence microscopy

As described in the study (Yang et al., 2007), phospho-STAT3's levels in MC3T3-E1 cells were characterized by the immunofluorescent assay. Concisely, MC3T3-E1 cells were stimulated with or without 5 ng/ml TNF-α for 2 hr and incubated with rabbit anti-P-Y705-STAT3 or rabbit IgG (Cell Signaling Technology, Beverly, MA). The cells subsequently were stained with 4,6-diamidino-2-phenylindole and phycoerythrin-anti-rabbit IgG and then examined under a fluorescence microscope.

2.8 | Alkaline phosphatase assay

The ALP staining was performed with a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime) according to the manufacturer's instructions. After treated for 7 days with culture medium altered every three days, the cells were harvested, washed with phosphate buffered saline (PBS; pH7.0), and incubated with a mixture of BCIP/NBT (Beyotime, Shanghai, China). The blue insoluble deposits in the visual field showed the zone with high ALP activity.

For ALP quantitative analysis, cells were lysed measured with a fluorometric detection kit using 4-methylumbelliferyl phosphate disodium substrate (Sigma, St. Louis, MO) following the manufacturer's instructions. The ALP activity of each sample was normalized by total protein concentration via BCA total protein quantify kit (Thermo Fisher Scientific).

2.9 | Assessment of cell mineralization

For the evaluation of mineralization, the culture medium was supplemented with 50 μg/ml ascorbic acid (Sigma) and 3 mM inorganic phosphate (NaH_2PO_4; Sigma) to promote matrix synthesis and mineralization process. At the indicated time points, cell plates were collected and the cells on each plate were fixed in 4% paraformaldehyde in PBS (v:v). Matrix mineralization was evaluated by Alizarin Red staining according to the previous report (Luppen, Smith, Spevak, Boskey, & Frenkel, 2010) and an Olympus microscope (Olympus, Tokyo, Japan) was used to perform microphotography.

2.10 | Statistical analysis

The software analysis of variance (SPSS 13.0, SPSS Inc., Chicago, IL) was used to find various treatments' effects after establishing that the data were normally distributed and of equivalent variances. The homogeneity of the variance of samples to be compared was examined employing Levene's test. The variance was tolerated to heterogeneous if $p > 0.05$, and the least significant difference (LSD) approach was used to carry out appropriate pairwise comparisons of treatment groups. Otherwise, Dunn's post hoc test was used to perform treatment groups' pairwise comparisons. Unless differently stated, the results are shown as the mean ± standard deviation and performed in triplicate and repeated no less than one time.

3 | RESULTS

3.1 | TNF-α upregulates ELP2 in MC3T3-E1 cells at early stages of osteoblast differentiation

We first analyzed the gene expression occurring during osteogenic differentiation inhibition induced by TNF-α using the established preosteoblastic MC3T3-E1 cell line, which has osteogenic potential in vitro. As shown in Figure 1a, at 3 days, TNF-α influenced the suppression of cell viability, indicated by a marked decrease in ALP activity, which is an early marker of osteoblast differentiation (Figure 1b). Furthermore, TNF-α decreased the osteogenic capacity of MC3T3-E1 cells to induce extracellular matrix mineralization (Figure 1c).

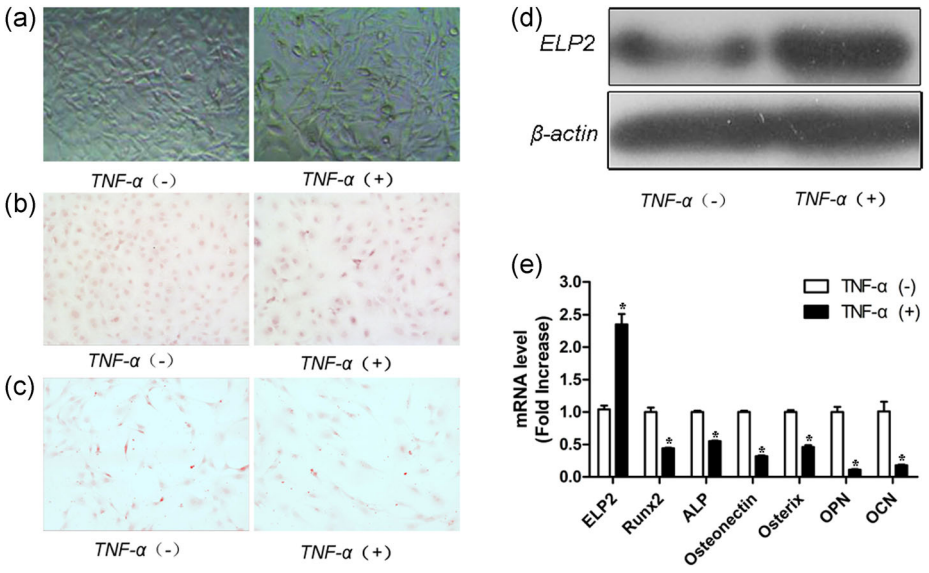

FIGURE 1 TNF-α upregulates ELP2 at the early stages of osteoblast differentiation in MC3T3-E1 cells. (a) Cell morphology was observed by microscopy; TNF-α (5 ng/ml) exerted an inhibitory effect on the growth of pre-osteoblastic MC3T3-E1 cells for 3 days. TNF-α decreased the ALP activity (b) and inhibited in vitro matrix mineralization (c), as showed by alizarin red staining in MC3T3-E1 cells at Day 3. (d) Western blot analysis confirmed that TNF-α induced the upregulation of ELP2 protein expression. (e) As evaluated by qRT-PCR, TNF-α promoted ELP2 but suppressed osteoblast gene mRNA expression at Day 3. TNF-α promoted ELP2 but suppressed osteoblast gene mRNA expression at Day 3 as evaluated by qRT-PCR. Data of at least three replicates are formulated to means ± *SD*. *$p < 0.05$ vs. unstimulated controls. ALP: alkaline phosphatase; ELP2: elongator complex protein 2; mRNA: messenger RNA; TNF-α: tumor necrosis factor-α; qRT-PCR: quantitative real-time polymerase chain reaction; SD: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

Western blot analysis confirmed that TNF-α promoted ELP2 protein expression in cells at Day 3 (Figure 1d). Remarkably, TNF-α increased ELP2 mRNA expression, and this increase was associated with decreased expression of Runx2, ALP, osteonectin, osterix, OPN, and OCN mRNA levels as revealed by qRT-PCR analysis (Figure 1e). These results imply that ELP2 is upregulated during the early stages of TNF-α-induced inhibition of osteoblast differentiation in MC3T3-E1 cells.

3.2 | TNF-α inhibits osteoblast differentiation in MC3T3-E1 cells through activation of STAT3

Next, we investigated the effect of TNF-α on the osteoblast differentiation of MC3T3-E1 cells. As reported by the precious report, the ALP expression level is a comparatively late marker of osteoblast differentiation used to monitor its progression. MC3T3-E1 cells were cultured in the medium with different concentrations of TNF-α (0, 2, 5, and 10 ng/ml) for 72 hr, and the proliferation these of each group was determined through ALP activity (Figure 2a). Osteoblast differentiation of MC3T3-E1 cells was hampered by TNF-α at high concentrations. The ALP activity of MC3T3-E1 cells treated with 5 ng/ml TNF-α was significantly reduced at the 24, 48, and 72 hr time points (Figure 2b).

Generally, TNF-α protein binds to its receptors, activate STAT3 and downstream signaling pathways to mediate cell proliferation. According to the result, the expression level of STAT3 was not significantly changed on account of TNF-α concentration in culture medium. But the phosphorylation of STAT3 in cells were significantly increased (Figure 2c,d). Therefore, our data demonstrate that TNF-α inhibits MC3T3-E1 cell osteoblast differentiation by activating STAT3.

3.3 | ELP2 inhibits osteogenic differentiation in MC3T3-E1 cells

We next determined the role of ELP2 in the early stages of osteoblast differentiation in MC3T3-E1 cells. A pcDNA3 expression vector encoding the full-length ELP2 sequence (pcDNA3-ELP2) was used to transduce cells along with an empty pcDNA3 vector as a control. Transduction with pcDNA3-ELP2 increased ELP2 protein expression in MC3T3-E1 cells (Figure 3a). We then investigated its effect on the basal expression of osteoblast markers, and qRT-PCR analysis showed that MC3T3-E1 cells transduced with pcDNA3-ELP2 exhibited lower Runx2, ALP, osteonectin, osterix, OPN, and OCN mRNA expression than those transduced with the empty pcDNA3 vector (Figure 3b). Furthermore, forced expression of ELP2 also decreased ALP activity and extracellular matrix mineralization in the cells (Figure 3c,d). These results demonstrate that forced ELP2 expression inhibits osteoblast marker gene expression and osteogenic differentiation in MC3T3-E1 cells.

3.4 | ELP2 silencing ameliorates TNF-α-induced osteogenic differentiation inhibition in MC3T3-E1 cells

To investigate the role of ELP2 in osteogenic differentiation inhibition by TNF-α in MC3T3-E1 cells, we next analyzed the effect of silencing

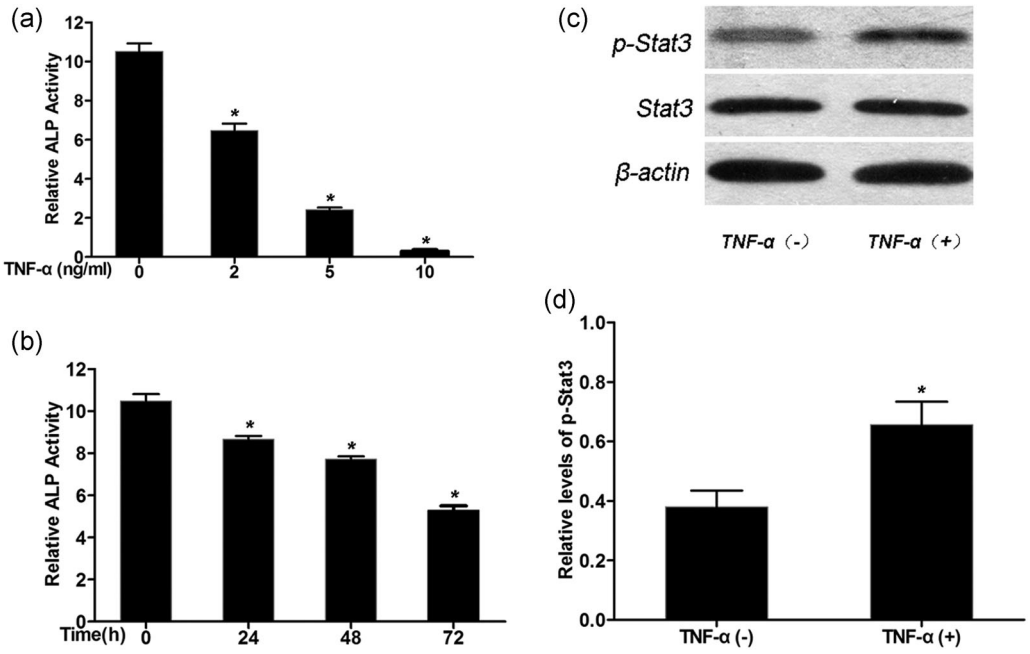

FIGURE 2 TNF-α inhibits MC3T3-E1 cell osteoblast differentiation by activating STAT3. MC3T3-E1 cells were incubated with culture medium with different concentrations of TNF-α for 72 hr (a) or 5 ng/ml of TNF-α for the indicated time periods (b). The intensity of osteoblast differentiation was determined via measuring the ALP activity. Cells were stimulated with 5 ng/ml TNF-α for 4 hr, and the relative levels of STAT3 expression and phosphorylation were determined by western blot analysis (c) and quantified by densitometry (d). Data are the means ± *SD* of at least three replicates. *$p < 0.05$ vs. unstimulated controls. ALP: alkaline phosphatase; TNF-α: tumor necrosis factor-α; SD: standard deviation; STAT3: signal transducer and activator of transcription 3

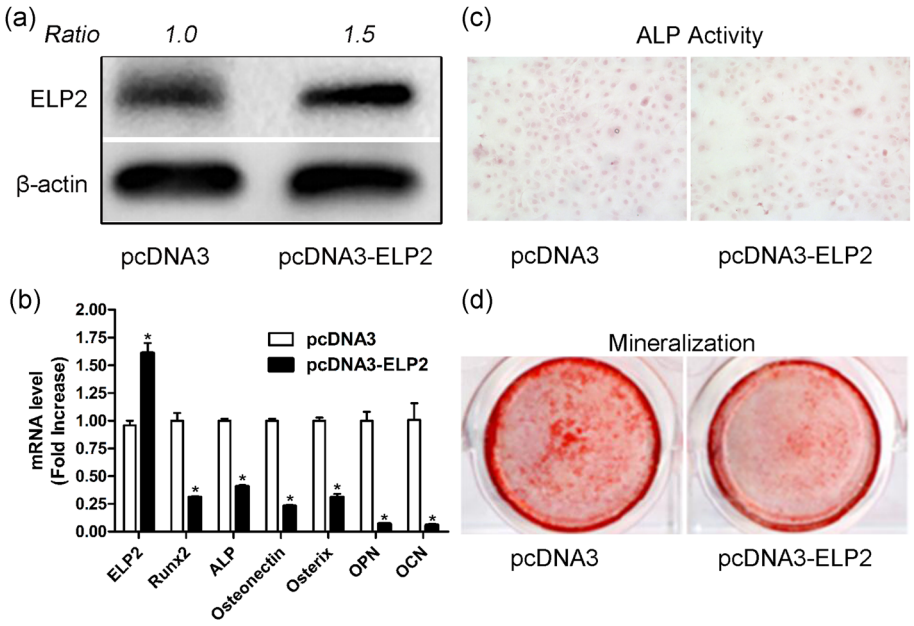

FIGURE 3 ELP2 reduces osteoblastic gene expression in MC3T3-E1 cells. (a) MC3T3-E1 cells were transduced with a vector encoding full-length ELP2 (pcDNA3-ELP2) or an empty pcDNA3 vector (pcDNA3), and ELP2 protein levels were revealed by western blot analysis. (b) Overexpression of ELP2 decreased Runx2, ALP, osteonectin, osterix, OPN, and OCN mRNA levels as determined by qRT-PCR in MC3T3-E1 cells. (c) ELP2 overexpression decreased ALP activity in MC3T3-E1 cells. (d) ELP2 overexpression inhibited in vitro matrix mineralization as revealed by alizarin red staining in MC3T3-E1 cells. Data are the means ± *SD* of at least three replicates. **p* < 0.05 vs. cells transduced with control lentiviral vector. ALP: alkaline phosphatase; ELP2: elongator protein complex 2; mRNA: messenger RNA; qRT-PCR: quantitative real-time polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

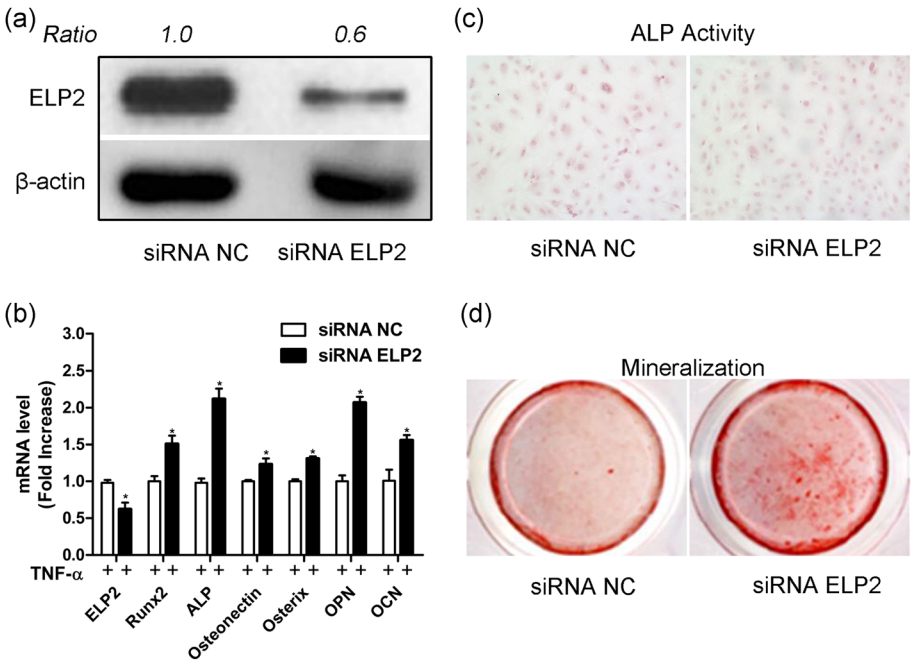

FIGURE 4 ELP2 silencing ameliorates the inhibition of osteogenic differentiation by TNF-α in MC3T3-E1 cells. (a) Cells were transduced with siRNA directed against ELP2 (ELP2 siRNA) or a negative control small interfering RNA (NC siRNA), and ELP2 protein levels were revealed by western blot analysis. (b) ELP2 silencing ameliorated TNF-α-inhibited osteoblastic gene expression in MC3T3-E1 cells. Cells transduced with ELP2 siRNA or NC siRNA were treated with TNF-α, and ELP2, Runx2, ALP, osteonectin, osterix, OPN, and OCN mRNA levels were evaluated by qRT-PCR. (c) The silencing of ELP2 promoted ALP activity in MC3T3-E1 cells treated with TNF-α. (d) The silencing of ELP2 promoted matrix mineralization as showed by alizarin red staining in MC3T3-E1 cells treated with TNF-α. Data are the means ± *SD* of at least three replicates. **p* < 0.05 vs. cells transduced with control lentiviral vector. ALP: alkaline phosphatase; ELP2: elongator complex protein 2; mRNA: messenger RNA; TNF-α: tumor necrosis factor α; qRT-PCR: quantitative real-time polymerase chain reaction; siRNA: small interfering RNA; SD: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

ELP2 expression. siRNA sequences were synthesized to inhibit ELP2 expression (ELP2 siRNA), and we analyzed the effect of ELP2 siRNA on the basal expression of osteoblast markers in comparison with a negative control siRNA (NC siRNA). As shown in Figure 4, MC3T3-E1 cells transduced with ELP2 siRNA expressed less ELP2 protein than those transduced with NC siRNA (Figure 4a). We then investigated the functional effect of ELP2 silencing on TNF-α-induced inhibition of MC3T3-E1 cell osteoblast differentiation. As shown in Figure 4b, silencing of ELP2 resulted in a marked increase in Runx2, ALP, osteonectin, osterix, OPN, and OCN mRNA expression in MC3T3-E1 cells. In addition, after treatment with TNF-α, ELP2 siRNA treatment resulted in a marked increase in ALP activity in cells (Figure 4c). Moreover, ELP2 silencing increased the osteogenic capacity of MC3T3-E1 cells cultured in the presence of TNF-α to induce extracellular matrix mineralization (Figure 4d). These results demonstrate that ELP2 knockdown promotes several TNF-α-inhibited osteoblast marker genes and osteogenic differentiation in the MC3T3-E1 cell line, suggesting that ELP2 is involved in the TNF-α-induced inhibition of MC3T3-E1 cell differentiation into osteoblasts.

3.5 | ELP2 promotes TNF-α-induced Janus kinase 2/STAT3 activation in MC3T3-E1 cells

To further understand the mechanisms underlying ELP2's action, we studied if ELP2 could facilitate the TNF-α-mediated Janus kinase 2 (JAK2)/STAT3 activation. According to the RNA interference result, the expression levels of JAK2 and STAT3 were similar in ELP2-silenced and control group, even boosted with a TNF-α stimulation. However, the levels of phospho-JAK2 and phospho-STAT3 in ELP2-silenced cells were clearly lower than those in the negative control group. And, the same phenomenon occurred when TNF-α was either absent or present (Figure 5a). According to the STAT3 transcriptional activity measurement, the activity of ELP2-silenced group was significantly lower than that of the negative control group and was not significantly affected with the presence of TNF-α (Figure 5b). In summary, these data indicated that ELP2 triggered the phosphorylation process of JAK2/STAT3. In addition, there was less intense anti-phospho-STAT3 staining in the nuclei of ELP2 siRNA-transfected cells than in those of control cells, even after TNF-α stimulation (Figure 5c).

4 | DISCUSSION

TNF-α is a pleiotropic cytokine that becomes elevated in chronic inflammatory states. The effects of TNF-α include slowing down osteogenic differentiation, which leads to bone dysplasia in long-term inflammatory microenvironments. A previous study of ELP2 function reported that overexpressing ELP2 leads to an upregulation of TNF-α-induced inhibition of osteoblast differentiation (Xu et al., 2015). Here, we identified a role for ELP2 in the early stages of TNF-α-induced osteogenic differentiation inhibition in MC3T3-E1 cells.

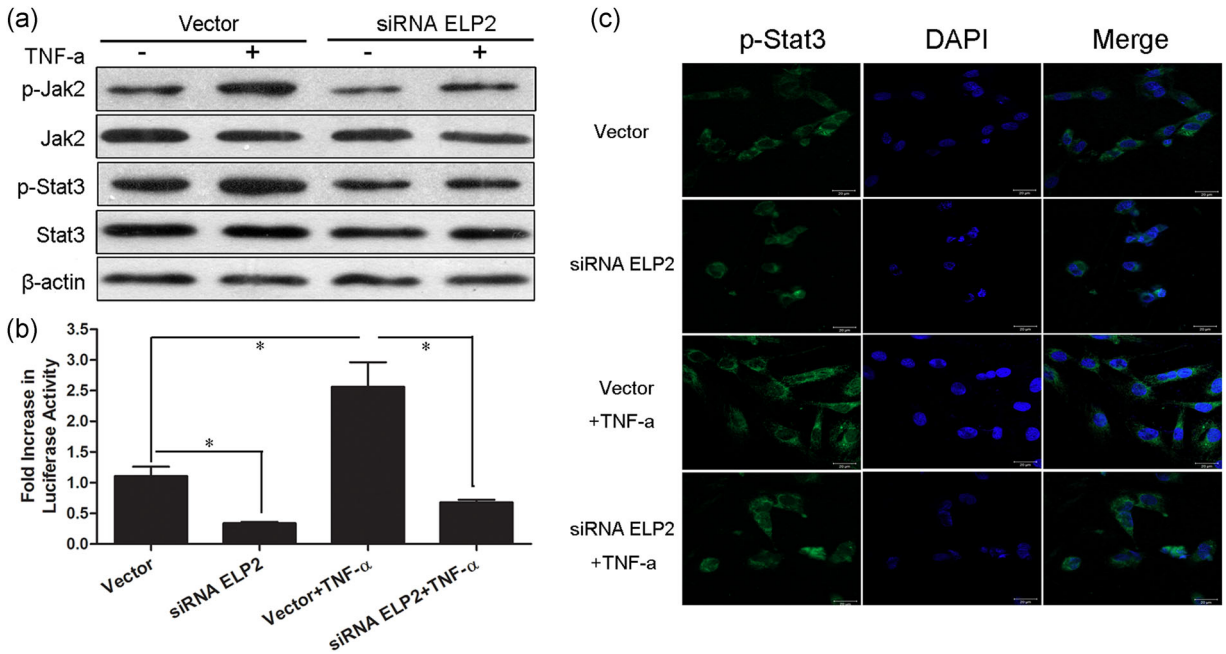

FIGURE 5 ELP2 promotes TNF-α-induced JAK2/STAT3 activation in MC3T3-E1 cells. The cells were stimulated with 5 ng/ml TNF-α or no TNF-α for 4 hr, the protein expression levels and phosphorylation level of STAT3, JAK2 were determined by western blot and immunofluorescent assays. In addition, STAT3 activation was detected by luciferase reporter assays following transfection of the indicated cells with relevant plasmids and stimulation with TNF-α. (a) Western blot analysis of JAK2/STAT3 activation. (b) Luciferase reporter assays of STAT3 activation. (c) Immunofluorescent analysis of STAT3 activation (magnification, ×400). The data shown are representative images or expressed as the means ± *SD* of different groups of cells from three separate experiments. *$p < 0.05$ vs. controls. ELP2: elongator complex protein 2; JAK2: Janus kinase 2; TNF-α: tumor necrosis factor-α; SD: standard devation; STAT3: signal transducer and activator of transcription 3 [Color figure can be viewed at wileyonlinelibrary.com]

Forced ELP2 expression inhibited osteogenic differentiation, whereas ELP2 silencing partly restored osteogenic differentiation even after TNF-α stimulation. Our results suggest that the TNF-α-induced inhibitory effect on osteoblastic differentiation is mediated by ELP2, which is associated with JAK2/STAT3 activation. These data extend our previous findings showing that ELP2 acts as an inhibitor of MC3T3-E1 cell differentiation into osteoblasts and suggest that ELP2 inhibits osteogenic differentiation in response to TNF-α through the STAT3 pathway.

ELP2 is the second largest subunit of the elongator complex and contains two WD40 propeller domains, which are involved in transcriptional elongation and act as histone acetyltransferase component of the RNA polymerase II (Pol II) holoenzyme. Dauden et al. (2017) showed that ELP2 promote the stability of the ELP123 sub-complex and integrates signals from different factors to modulate elongation activity. The report of Wang et al. also demonstrated that the conserved loop regions in ELP2 protein are a vital factor to the elongation function (Wang et al., 2013). Consequently, ELP2 plays an essential role in conferring total enzyme activity and occupies a significant role in downstream regulation. Our previous research on ELP2 function reported that overexpressing ELP2 results in the upregulation of TNF-α-induced inhibition of osteoblast differentiation (Xu et al., 2015). In the present study, we used preosteoblastic MC3T3-E1 cells to demonstrate that ELP2 is upregulated significantly during the early stages of TNF-α-induced inhibition of osteoblast differentiation compared with its expression in non-TNF-α-stimulated cells and that ELP2 expression is negatively associated with the osteogenic capacity and extracellular matrix mineralization of MC3T3-E1 cells. We also found that ELP2 overexpression inhibited spontaneous MC3T3-E1 cell osteogenic differentiation. ELP2 may be a positive regulator of the inflammatory microenvironment in MC3T3-E1 cells, which is negatively associated with osteoblast differentiation.

A growing body of evidence suggests that an inflammatory environment has an adverse effect on the recovery of bone tissue (H. Huang et al., 2011; Novack & Mbalaviele, 2016; Robaszkiewicz et al., 2016). Among the inflammatory factors, TNF-α may affect osteoclastogenesis and bone formation (Kong et al., 2013; Qin et al., 2015). Inflammation may have both positive and negative effects on bone regeneration in various physiological and pathological processes. In contrast to the classical effects of inflammatory factors on bone loss, work from several laboratories has revealed that TNF-α may increase osteogenesis (Hess, Ushmorov, Fiedler, Brenner, & Wirth, 2009; Z. Lu, Wang, Dunstan, & Zreiqat, 2012; Mountziaris, Tzouanas, & Mikos, 2010). A prominent difference between this discordance may lie not only in the differentiation state of the starting cells but also in the TNF-α concentration. Some studies have demonstrated that low-concentration TNF-α (< or = 1 ng/ml) moderately promotes osteoblast differentiation (Daniele et al., 2017;

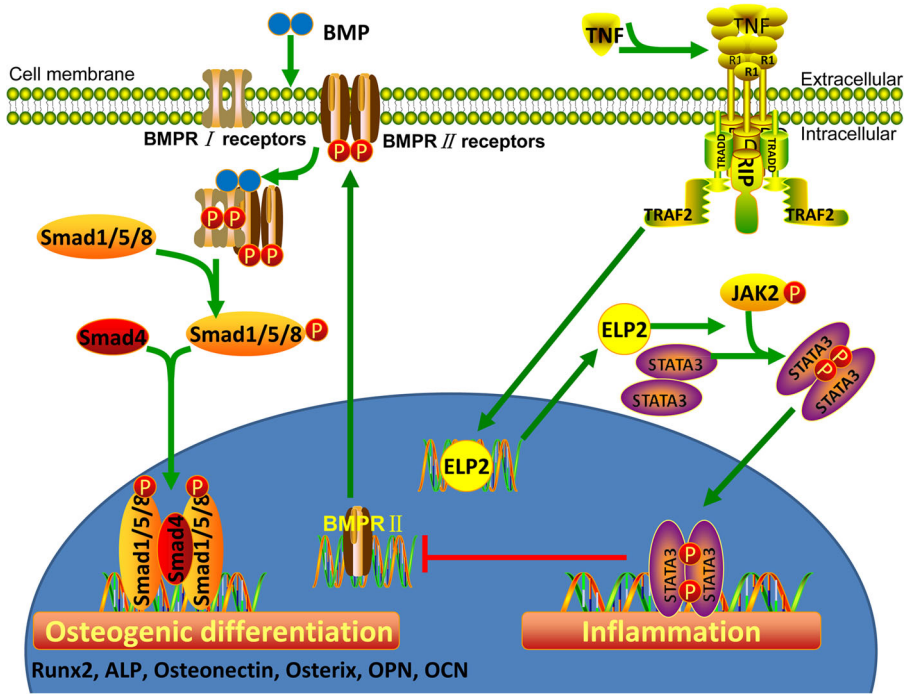

FIGURE 6 A proposed model by which ELP2 mediates TNF-α-induced osteogenic differentiation inhibition in MC3T3-E1 cells. ELP2 upregulation induced by TNF-α increases STAT3 activation, resulting in decreased Runx2 and osteoblast differentiation gene expression in MC3T3-E1 cells. When ELP2 is upregulated after TNF-α stimulation, JAK2 is phosphorylated. This phosphorylation leads to STAT3 phosphorylation and subsequent dimerization of this molecule. The phosphorylated STAT3 dimer translocates to the nucleus and activates the transcription of genes associated with inflammation, led to the inhibition of BMPR2 expression and block BMP-2-induced osteoblast differentiation. ALP2: alkaline phosphatase; BMPR-2: bone morphogenetic protein receptor 2; ELP2: elongator complex protein 2; TNF-α: tumor necrosis factor-α; STAT3: signal transducer and activator of transcription 3 [Color figure can be viewed at wileyonlinelibrary.com]

H. Huang et al., 2011). In this study, we found that high-concentration TNF-α mainly had negative effects on osteogenic differentiation, mostly consistent with the previous reports (Gilbert et al., 2000; Tsukasaki et al., 2011). We also found that TNF-α inhibited MC3T3-E1 cell osteogenic differentiation, which was associated with receptor-mediated downstream STAT3 activation. Our data are consistent with previous findings (Nan et al., 2017) and demonstrate that TNF-α inhibits MC3T3-E1 cell osteogenic differentiation through its receptor. Interestingly, we found that ELP2 silencing ameliorated the inhibition of osteogenic differentiation by TNF-α in MC3T3-E1 cells. ELP2 silencing may inhibit inflammation in MC3T3-E1 cells, thereby promoting osteogenic differentiation.

Through its TNF-α receptor/TNFR2, TNF-α can activate the downstream STAT3 pathway in inflammation-associated diseases (Hamilton, Simmons, Ding, Van, & Lund, 2011). STAT3 is an important transcription factor that plays crucial roles in immune regulation, inflammation, cell proliferation, transformation, and other physiological processes (Y. Huang et al., 2015). We found that high levels of JAK2/STAT3 activation were positively regulated by exogenous TNF-α, which was consistent with previous reports (Agrawal, Gollapudi, Su, & Gupta, 2011; Chen et al., 2018), and subsequently suppressed the osteogenic differentiation of MC3T3-E1 cells in pathological processes. In addition, we also found that ELP2 silencing inhibited spontaneous and TNF-α-promoted STAT3 activation in MC3T3-E1 cells, accompanied by increased Runx2, ALP, osteonectin, osterix, OPN, and OCN expression, enhanced matrix mineralization and lower levels of phospho-STAT3 and phospho-JAK2 in MC3T3-E1 cells. Our findings indicate that the inhibition of TNF-α-induced STAT3 activation can promote MC3T3-E1 cell osteogenic differentiation (He, Yang, Isales, & Shi, 2012; Przekora & Ginalska, 2017). The JAK2/STAT3 signaling pathway is one of the most described pathways that is known to negatively regulate osteoblast differentiation (Matsushita et al., 2014). In contrast, a previous study (Xie et al., 2018) demonstrated that IL-6 activated the downstream STAT3 signaling pathway, which promotes osteogenic differentiation in bone marrow-derived mesenchymal stem cells in physiological processes. We conclude that controversies regarding the roles of STAT3 activation in osteoblastic differentiation may have resulted from differences in the cell source and differences in experimental conditions in vitro. Interestingly, we found that when TNF-α was added, ELP2 and STAT3 were elevated, indicating that ELP2 may regulate Runx2, ALP, osteonectin, osterix, OPN, and OCN expression to reduce MC3T3-E1 cell osteogenic differentiation and matrix mineralization. It can be seen from these data that ELP2 is involved in osteoblast differentiation via STAT3. The activation of STAT3 also leads to a downregulation of bone morphogenetic protein receptor 2 (BMPR2; Brock et al., 2009; Meloche et al., 2013), which is the central receptor of BMP-2 in osteoblast differentiation via the BMP-2/Smad1/5/8 pathways (Haversath, Catelas, Li, Tassemeier, & Jäger, 2012; Liou et al., 2015). Another study showed that TNF-α inhibits BMPR2 expression (Singhatanadgit, Salih, & Olsen, 2006). Based on our results, we propose a model in which ELP2 is upregulated by TNF-α and activates STAT3, which then inhibits BMPR2 expression to block BMP-2; therefore, BMP-2-induced osteoblast differentiation is impaired in MC3T3-E1 cells (Figure 6). Conceivably, modulation of ELP2 expression or therapeutic treatment with ELP2 may be a promising strategy to control inflammatory osteophytosis disorders in the clinic. However, several limitations still exist in this study, including the lack of understanding of the regulatory mechanism of TNF-α signaling in inhibiting osteoblast differentiation. In addition, the functions of STAT3 during osteogenic differentiation in vitro are still ambiguous. These limitations should be addressed in future studies.

In conclusion, our data indicate that high concentrations of TNF-α inhibit osteoblastic differentiation accompanied by increased ELP2 expression and STAT3 activation in preosteoblastic MC3T3-E1 cells. Furthermore, ELP2 is involved in osteoblast differentiation and acts downstream of TNF-α. Forced ELP2 expression inhibits osteogenic differentiation in MC3T3-E1 cells, and ELP2 silencing ameliorates the inhibition of osteogenic differentiation in MC3T3-E1 cells, even after TNF-α stimulation. In addition, we found that the TNF-α-induced inhibitory effect on osteoblastic differentiation is mediated by ELP2 and associated with STAT3 activation. Taken together, these results suggest that ELP2 is upregulated at MC3T3-E1 cell differentiation into osteoblasts and inhibits osteogenic differentiation in response to TNF-α through STAT3 activation.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Fundation for Youths of China (No. 81501902), Guangdong Province Natural Science Foundation (nos 2014A030310451, 2017A030313736), Science and Technology Planning Project of Guangzhou (No. 201804010226), Science Foundation of Guangdong Second Provincial General Hospital (No. YQ2016-003), National Natural Science Fundation of China (No. 81560368), Science and Technology Assistance Project of Xinjiang Province (No. 2018E02056), and Natural Science Foundation of Xinjiang Province (No. 2018D01C014). In addition, Chang-Peng Xu thanks the inimitable care and support of Xiao-Jie Zheng over the years. I love you. Will you spend the rest of your life with me? The authors thank LetPub (www.letpub.com) for providing linguistic assistance during the preparation of this manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

C. -P. X. and H.-T. S. wrote the manuscript. C.-P. X., and Y. Q. critically revised the manuscript. All authors read and approved the final manuscript.

ORCID

Chang-Peng Xu http://orcid.org/0000-0001-6349-0842

REFERENCES

Agrawal, S., Gollapudi, S., Su, H., & Gupta, S. (2011). Leptin activates human B cells to secrete TNF-α, IL-6, and IL-10 via JAK2/STAT3 and p38MAPK/ERK1/2 signaling pathway. *Journal of Clinical Immunology*, *31*(3), 472–478.

Almeida, M., Laurent, M. R., Dubois, V., Claessens, F., O'Brien, C. A., Bouillon, R., … Manolagas, S. C. (2017). Estrogens and androgens in skeletal physiology and pathophysiology. *Physiological Reviews*, *97*(1), 135–187.

Brock, M., Trenkmann, M., Gay, R. E., Michel, B. A., Gay, S., Fischler, M., & Huber, L. C. (2009). Interleukin-6 modulates the expression of the bone morphogenic protein receptor TYpe II through a novel STAT3–microRNA cluster 17/92 pathway. *Circulation Research*, *104*(10), 1184–1191.

Chen, X., Nie, X., Mao, J., Zhang, Y., Yin, K., & Jiang, S. (2018). Perfluorooctanesulfonate induces neuroinflammation through the secretion of TNF-α mediated by the JAK2 / STAT3 pathway. *Neurotoxicology*, *66*, 32–42.

Collum, R. G., Brutsaert, S., Lee, G., & Schindler, C. (2000). A stat3-interacting protein (StIP1) regulates cytokine signal transduction. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(18), 10120–10125.

Daniele, S., Natali, L., Giacomelli, C., Campiglia, P., Novellino, E., Martini, C., & Trincavelli, M. L. (2017). Osteogenesis is improved by low tumor necrosis factor alpha concentration through the modulation of gs-coupled receptor signals. *Molecular and Cellular Biology*, *37*(8), e00442–16.

Dauden, M. I., Kosinski, J., Kolaj, R. O., Desfosses, A., Ori, A., Faux, C., & Müller, C. W. (2017). Architecture of the yeast elongator complex. *EMBO Reports*, *18*(2), 264–279.

Gilbert, L., He, X., Farmer, P., Boden, S., Kozlowski, M., Rubin, J., & Nanes, M. S. (2000). Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology*, *141*(11), 3956–3964.

Glatt, S., Séraphin, B., & Müller, C. W. (2012). Elongator: Transcriptional or translational regulator? *Transcription*, *3*(6), 273–276.

Hamilton, K. E., Simmons, J. G., Ding, S., Van, L. L., & Lund, P. K. (2011). Cytokine-induction of tumor necrosis factor receptor 2 (TNFR2) is mediated by STAT3 in colon cancer cells. *Molecular Cancer Research*, *9*(12), 1718–1731.

Haversath, M., Catelas, I., Li, X., Tassemeier, T., & Jäger, M. (2012). PGE_2 and BMP-2 in bone and cartilage metabolism: 2 intertwining pathways. *Canadian Journal of Physiology & Pharmacology*, *90*(11), 1434–1445.

Hawkes, N. A., Otero, G., Winkler, G. S., Marshall, N., Dahmus, M. E., Krappmann, D., & Svejstrup, J. Q. (2002). Purification and characterization of the human elongator complex. *Journal of Biological Chemistry*, *277*, 3047–3052.

He, L., Yang, N., Isales, C. M., & Shi, X. M. (2012). Glucocorticoid-induced leucine zipper (GILZ) antagonizes tumor necrosis factor-alpha (TNF-α) inhibition of mesenchymal stem cell osteogenic differentiation. *PLOS One*, *7*(3), e31717.

Hess, K., Ushmorov, A., Fiedler, J., Brenner, R. E., & Wirth, T. (2009). TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. *Bone*, *45*(2), 367–376.

Huang, H., Zhao, N., Xu, X., Xu, Y., Li, S., Zhang, J., & Yang, P. (2011). Dose-specific effects of tumor necrosis factor alpha on osteogenic differentiation of mesenchymal stem cells. *Cell Proliferation*, *44*(5), 420–427.

Huang, Y., Huang, X., Yang, Y., Wang, W., Yu, Y., & Qin, Q. (2015). Involvement of fish signal transducer and activator of transcription 3 (STAT3) in nodavirus infection induced cell death. *Fish & Shellfish Immunology*, *43*(1), 241–248.

Kaneki, H., Guo, R., Chen, D., Yao, Z., Schwarz, E. M., Zhang, Y. E., & Xing, L. (2006). Tumor necrosis factor promotes Runx2 degradation through up-regulation of Smurf1 and Smurf2 in osteoblasts. *Journal of Biological Chemistry*, *281*(7), 4326–4333.

Karlsborn, T., Tükenmez, H., Mahmud, A. K., Xu, F., Xu, H., & Byström, A. S. (2014). Elongator, a conserved complex required for wobble uridine modifications in eukaryotes. *RNA Biology*, *11*(12), 1519–1528.

Kong, X., Liu, Y., Ye, R., Zhu, B., Zhu, Y., Liu, X., & Jin, Y. (2013). GSK3beta is a checkpoint for TNF-alpha-mediated impaired osteogenic differentiation of mesenchymal stem cells in inflammatory microenvironments. *Biochimica et Biophysica Acta/General Subjects*, *1830*(11), 5119–5129.

Li, W., Yu, B., Li, M., Sun, D., Hu, Y., Zhao, M., & Hou, S. (2010). NEMO-binding domain peptide promotes osteoblast differentiation impaired by tumor necrosis factor alpha. *Biochemical and Biophysical Research Communications*, *391*(2), 1228–1233.

Liou, S. F., Hsu, J. H., Chu, H. C., Lin, H. H., Chen, I. J., & Yeh, J. L. (2015). KMUP-1 promotes osteoblast differentiation through cAMP and cGMP pathways and signaling of BMP-2/Smad1/5/8 and Wnt/β-catenin. *Journal of Cellular Physiology*, *230*(9), 2038–2048.

Lu, S., Fan, H. W., Li, K., & Fan, X. D. (2018). Suppression of Elp2 prevents renal fibrosis and inflammation induced by unilateral ureter obstruction (UUO) via inactivating Stat3-regulated TGF-β1 and NF-κB pathways. *Biochemical and Biophysical Research Communications*, *501*(2), 400–407.

Lu, Z., Wang, G., Dunstan, C. R., & Zreiqat, H. (2012). Short-term exposure to tumor necrosis factor-alpha enables human osteoblasts to direct adipose tissue-derived mesenchymal stem cells into osteogenic differentiation. *Stem Cells and Development*, *21*(13), 2420–2429.

Luppen, C. A., Smith, E., Spevak, L., Boskey, A. L., & Frenkel, B. (2010). Bone morphogenetic protein-2 restores mineralization in glucocorticoid-inhibited MC3T3-E1 osteoblast cultures. *Journal of Bone & Mineral Research*, *18*(7), 1186–1197.

Matsushita, K., Itoh, S., Ikeda, S., Yamamoto, Y., Yamauchi, Y., & Hayashi, M. (2014). LIF/STAT3/SOCS3 signaling pathway in murine bone marrow stromal cells suppresses osteoblast differentiation. *Journal of Cellular Biochemistry*, *115*(7), 1262–1268.

Mcbridegagyi, S. H., Mckenzie, J. A., Buettmann, E. G., Gardner, M. J., & Silva, M. J. (2015). Bmp2 conditional knockout in osteoblasts and endothelial cells does not impair bone formation after injury or mechanical loading in adult mice. *Bone*, *81*, 533–543.

Meloche, J., Courchesne, A., Barrier, M., Carter, S., Bisserier, M., Paulin, R., & Bonnet, S. (2013). Critical role for the advanced glycation end-products receptor in pulmonary arterial hypertension etiology. *Journal of the American Heart Association Cardiovascular & Cerebrovascular Disease*, *2*(1), e005157.

Mountziaris, P. M., Tzouanas, S. N., & Mikos, A. G. (2010). Dose effect of tumor necrosis factor-alpha on in vitro osteogenic differentiation of mesenchymal stem cells on biodegradable polymeric microfiber scaffolds. *Biomaterials*, *31*(7), 1666–1675.

Mukai, T., Otsuka, F., Otani, H., Yamashita, M., Takasugi, K., Inagaki, K., & Makino, H. (2007). TNF-alpha inhibits BMP-induced osteoblast differentiation through activating SAPK/JNK signaling. *Biochemical and Biophysical Research Communications*, *356*(4), 1004–1010.

Nan, J., Hu, H., Sun, Y., Zhu, L., Wang, Y., Zhong, Z., & Wang, J. (2017). TNFR2 stimulation promotes mitochondrial fusion via Stat3- and NF-kB–dependent activation of OPA1 expression. *Circulation Research*, *121*(4), 392–410.

Novack, D. V., & Mbalaviele, G. (2016). Osteoclasts-key players in skeletal health and disease. *Microbiology Spectrum*, *4*(3).

Przekora, A., & Ginalska, G. (2017). Chitosan/β-1,3-glucan/hydroxyapatite bone scaffold enhances osteogenic differentiation through TNF-α-mediated mechanism. *Materials Science & Engineering C*, *73*, 225–233.

Qin, Z., Fang, Z., Zhao, L., Chen, J., Li, Y., & Liu, G. (2015). High dose of TNF-α suppressed osteogenic differentiation of human dental pulp

stem cells by activating the Wnt/β-catenin signaling. *Journal of Molecular Histology*, *46*(4-5), 409–420.

Robaszkiewicz, A., Qu, C., Wisnik, E., Ploszaj, T., Mirsaidi, A., Kunze, F. A., & Hottiger, M. O. (2016). ARTD1 regulates osteoclastogenesis and bone homeostasis by dampening NF-κB-dependent transcription of IL-1β. *Scientific Reports*, *6*, 21131.

Singhatanadgit, W., Salih, V., & Olsen, I. (2006). Bone morphogenetic protein receptors and bone morphogenetic protein signaling are controlled by tumor necrosis factor-alpha in human bone cells. *International Journal of Biochemistry and Cell Biology*, *38*(10), 1794–1807.

Son, Y. O., Kook, S. H., Choi, K. C., Jang, Y. S., Choi, Y. S., Jeon, Y. M., & Lee, J. C. (2008). Quercetin accelerates TNF-alpha-induced apoptosis of MC3T3-E1 osteoblastic cells through caspase-dependent and JNK-mediated pathways. *European Journal of Pharmacology*, *579*(1-3), 26–33.

Tsialogiannis, E., Polyzois, I., Oak, T. Q., Pavlou, G., Tsiridis, E., Heliotis, M., & Tsiridis, E. (2009). Targeting bone morphogenetic protein antagonists: In vitro and in vivo evidence of their role in bone metabolism. *Expert Opinion on Therapeutic Targets*, *13*(1), 123–137.

Tsukasaki, M., Yamada, A., Suzuki, D., Aizawa, R., Miyazono, A., Miyamoto, Y., & Kamijo, R. (2011). Expression of POEM, a positive regulator of osteoblast differentiation, is suppressed by TNF-α. *Biochemical & Biophysical Research Communications*, *410*(4), 766–770.

Wang, Y., An, C., Zhang, X., Yao, J., Zhang, Y., Sun, Y., & Mou, Z. (2013). The arabidopsis elongator complex subunit2 epigenetically regulates plant immune responses. *The Plant Cell*, *25*(2), 762–776.

Xie, Z., Tang, S., Ye, G., Wang, P., Li, J., Liu, W., & Shen, H. (2018). Interleukin-6/interleukin-6 receptor complex promotes osteogenic differentiation of bone marrow-derived mesenchymal stem cells. *Stem Cell Research & Therapy*, *9*(1), 13.

Xu, C. P., Li, X., Hu, Y. J., Cui, Z., Wang, L., Liang, L., & Yu, B. (2015). Quantitative proteomics reveals ELP2 as a regulator to the inhibitory effect of TNF-α on osteoblast differentiation. *Journal of Proteomics*, *114*, 234–246.

Yamazaki, M., Fukushima, H., Shin, M., Katagiri, T., Doi, T., Takahashi, T., & Jimi, E. (2009). Tumor necrosis factor alpha represses bone morphogenetic protein (BMP) signaling by interfering with the DNA binding of Smads through the activation of NF-kappaB. *Journal of Biological Chemistry*, *284*(51), 35987–35995.

Yang, Y., Zhou, H., Yang, Y., Li, W., Zhou, M., Zeng, Z., & Li, G. (2007). Lipopolysaccharide (LPS) regulates TLR4 signal transduction in nasopharynx epithelial cell line 5–8F via NFkappaB and MAPKs signaling pathways. *Molecular Immunology*, *44*(5), 984–992.

Yoshimura, A. (2006). Signal transduction of inflammatory cytokines and tumor development. *Cancer Prevention Research*, *97*(6), 439–447.

Zhang, X., Xiao, W., Wang, L., Tian, Z., & Zhang, J. (2011). Deactivation of signal transducer and activator of transcription 3 reverses chemotherapeutics resistance of leukemia cells via down-regulating P-gp. *PLOS One*, *6*(6), e20965.

How to cite this article: Xu C-P, Sun H-T, Yang Y-J, et al. ELP2 negatively regulates osteoblastic differentiation impaired by tumor necrosis factor α in MC3T3–E1 cells through STAT3 activation. *J Cell Physiol*. 2019;234:18075–18085. https://doi.org/10.1002/jcp.28440