Tanshinone IIA inhibits osteoclast differentiation through down-regulation of c-Fos and NFATc1

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Abstract

Bone is a dynamic tissue that is regulated by the activity of bone-resorbing osteoclasts and bone-forming osteoblasts. Excessive osteoclast forma-tion causes diseases such as osteoporosis and rheumatoid arthritis. Natural substances may be useful as therapeutic drugs to prevent many di-seases in humans because they avoid the many side effects of treatment with chemical compounds. Here we show that tanshinone IIA isolated from *Salvia miltiorrhiza* Bunge inhibits the receptor activator of NF-κB ligand (RANKL)-mediated osteoclast differen-tiation of osteoclast precursors. Tanshinone IIA suppressed the expression levels of c-Fos and NFATc1 induced by RANKL. However, retrovirus-mediated overexpression of c-Fos induced the ex-pression of NFATc1 despite the presence of tans-hinone IIA and reversed the inhibitory effect of tanshinone IIA on osteoclast differentiation. Also, the introduction of osteoclast precursors with the NFATc1 retrovirus led to osteoclast differentiation in the presence of tanshinone IIA. Our results suggest that tanshinone IIA may have a role as a therapeutic drug in the treatment of bone disease such as osteoporosis.

Keywords: osteoclasts; tanshinone; Salvia miltior-rhiza; drugs, Chinese herbal; osteoporosis; RANK ligand

Introduction

The balance between bone-resorbing osteoclasts and bone-forming osteoblasts is important to bone homeostasis. Many factors tightly regulate bone resorption and bone formation *in vivo* to maintain bone homeostasis (Suda *et al*., 1999; Boyle *et al*., 2003). Bone erosion through excessive osteoclast formation is associated with alterations including estrogen deficiency and increases in inflammatory cytokines (Abu-Amer *et al*., 1997; Miyaura *et al*., 2003) and in turn causes bone diseases such as rheumatoid arthritis, periodontal disease, osteoporo-sis, and periprosthetic osteolysis. In addition, under certain conditions such as aging or prolonged smok-ing, bone loss is caused by a defect in the bone-forming osteoblasts (Merkel *et al*., 1999; Liu *et al*., 2001; Redlich *et al*., 2002). Most therapeutic drugs currently used to treat bone disease inhibit oste-oclast formation or bone resorption. Consequently, there is a need for effective therapeutic drugs that improve the quality and quantity of bone.

Osteoclasts, which are the cells primarily respon-sible for bone resorption, are of hamatopoietic origin. Osteoclast differentiation is regulated by multiple processes through differential gene expression, one of which is characterized by the fusion of osteoclast precursors. Osteotropic agents including, IL-1, IL-6, IL-11, IL-15, IL-17, TNF-α, PGE2 and PTH cause bone loss by increasing osteoclast formation (Suda *et al*., 1999; Hofbauer *et al*., 2000). Receptor ac-tivator of NF-κB ligand (RANKL), which is an essential osteoclastogenic cytokine, recruits TNF receptor-associated factor (TRAF) proteins through binding to its receptor, RANK, on the surface of osteoclast precursors. TRAFs activate various sig-naling pathways, including c-Src, PI3-kinase/Akt, and MAPK (Anderson *et al*., 1997; Lee and Kim, 2003). Several transcription factors, including NF-κB, c-Fos, NFATc1, PU1 and MITF, are activated by RANKL and are important for osteoclast differen-tiation (Teitelbaum *et al*., 2003). In particular, ex-pression of c-Fos induced by RANKL plays an

essential role in the initiation of osteoclast differentiation. c-Fos causes the expression of NFATc1 during RANKL-mediated osteoclast differentiation (Matsuo *et al.*, 2004). NFATc1 can induce osteoclast differentiation in the absence of RANKL. Thus, NFATc1 is an essential factor in osteoclast differentiation (Takayanagi *et al.*, 2002).

Tanshinones are the major component of Danshen, the dried roots of *Salvia miltiorrhiza* Bunge (Labiatae) that is used as a drug in traditional Chinese medicine (Hazra *et al.*, 2004; Hur *et al.*, 2005). Tanshinones have been widely used to treat menorrhalgia, insomnia, coronary disease, and cardiovascular disease (Takahashi *et al.*, 2002; Han *et al.*, 2005). Furthermore, tanshinone IIA has been shown to be a potent antioxidant and to have anticancer activity against various cancer cells (Wu *et al.*, 1998; Wang *et al.*, 2005). Although tanshinone IIA has been used as a medicinal agent in the treatment of many diseases, its role in osteoclast-related bone disease remains unknown.

We showed previously that tanshinone IIA greatly inhibits osteoclast differentiation and suppresses bone resorption through disruption of the actin ring (Kim *et al.*, 2004). Here we examine the precise inhibitory mechanism of tanshinone IIA on RANKL-induced osteoclast differentiation. This study shows that tanshinone IIA prevents osteoclast differentiation by inhibiting c-Fos and NFATc1 expression.

Materials and Methods

Reagents

Tanshinone IIA was isolated from Danshen as described previously (Kim *et al.*, 2004). Recombinant human RANKL and macrophage CSF (M-CSF) were purchased from PeproTech EC (London, England). Antibody to c-Fos was obtained from Cell Signaling Technology (Beverly, MA). Antibodies to NFATc1 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). pMX-IRES-EGFP, pMX-c-Fos-IRES-EGFP, and pMX-NFATc1-IRES-EGFP vectors were kindly provided by Dr. Nacksung Kim (University of Chonnam).

Isolation of osteoclast precursors and osteoclastogenesis

Osteoclast precursors were prepared as previously described with some modification (Park *et al.*, 2005). Briefly, bone marrow cells were obtained from the tibia and femur of 5-week-old male ICR mice. Bone marrow cells were suspended in α-MEM (Welgene) containing 10% FBS (Gibco BRL) and antibiotics (Sigma) and were cultured for 1 day in the presence of M-CSF (10 ng/ml). After 1 day, nonadherent cells were seeded on a 10-cm bacterial culture dishes and cultured for 3 days in the presence of M-CSF (30 ng/ml) and further adherent cells were used as osteoclast precursors. For the osteoclast differentiation assay, osteoclast precursors were cultured for 4 days with M-CSF (30 ng/ml) and RANKL (50 ng/ml) in the presence or absence of tanshinone IIA (10 μg/ml). Alternatively, calvarial osteoblasts were isolated from 1-day-old ICR mouse as described previously (Kwak *et al.*, 2004). Briefly, mouse calvariae were obtained from pericranium and washed with HBSS containing antibiotics. Calvariae were digested 5 times for 15 min in 0.1% collagenase and 0.2% dispase. Cells were cultured for 3 days and adherent cells were used as osteoblasts. Osteoblasts and bone marrow cells were cocultured for 6 days with TNF-α (20 ng/ml), IL-1α (10 ng/ml), or LPS (1 μg/ml) in the presence or absence of tanshinone IIA (10 μg/ml).

Reverse transcription (RT)-PCR analysis

For RT-PCR assays, total RNA was isolated by using the TRI reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two micrograms of total RNA from each sample was reverse transcribed in a mixture containing oligo dT primer, dNTP, reverse transcriptase buffer, RNase out, DTT, and Superscript II reverse transcriptase (Invitrogen). cDNA (1 μl) was amplified by PCR to generate the genes listed as follows: RANK sense, 5'-CACAGACAAATGCAAACCTTG-3; RANK antisense, 5'-GTGTTCTGGAACCTATCTTCCTCC-3'; c-Fms sense, 5'-AGCTCTCAGTACTTCAGGGC-3'; c-Fms antisense, 5'-CAAAGGCACCGGCTCCTAGA-3'; c-Fos sense, 5'-CTGGTGCAGCCCACTCTGGTC-3'; c-Fos antisense, 5'-CTTTCAGCAGATTGGCAATCTC-3'; NFATc1 sense, 5'-CAACGCCCTGACCACCGATAG-3'; NFATc1 antisense, 5'-GGCTGCCTTCCGTCTCATAGT-3'. PCR amplification was performed for 20-25 cycles at 94°C for 30 s, 56-58°C for 30 s, and 72°C for 30 s, followed by a 7 min at 72°C. PCR product were separated on a 1% agarose gels and visualized by staining with ethidium bromide. Densitometric values for each band were quantified by using the Image Pro-plus program version 4.0 (Media Cybernetics).

Immunoblotting

Cells were lysed in lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, and 1% deoxycholate) containing protease inhibitors. Cell lysates were centrifuged at $16,000 \times g$ for 20 min, and total protein was measured with a DC Protein Assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were electro-

phoresed by 10-15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Protein levels were analyzed by immunoblotting with antibodies to c-Fos, NFATc1, and actin that were detected by using enhanced chemiluminescent reagents.

Retrovirus preparation

For retrovirus preparation, Plat E packaging cells were transiently transfected with pMX-IRES-EGFP, pMX-c-Fos-IRES-EGFP, or pMX-NFATc1-IRES-EGFP vectors by using Lipofectamine 2000 as recommended by the manufacturer. Six to eight hour after transfection, the medium was replaced with DMEM containing 10% FBS and antibiotics. Culture super-

natant fluid containing retrovirus was collected 48 h after transfection and was used as a retrovirus.

Luciferase assay

NFATc1 luciferase reporter gene was kindly provided by Dr. Nacksung Kim (University of Chonnam). HEK 293T cells were plated in 24-well plates and transiently transfected with RANK and the AP-1 or NFATc1 leuciferase reporter genes using Lipofectamine 2000 and incubated for 24 h with or without tanshinone IIA. The cells were lysed with lysis buffer, and the luciferase activities were measured using a luciferase reporter assay system (Promega, Madison, WI).
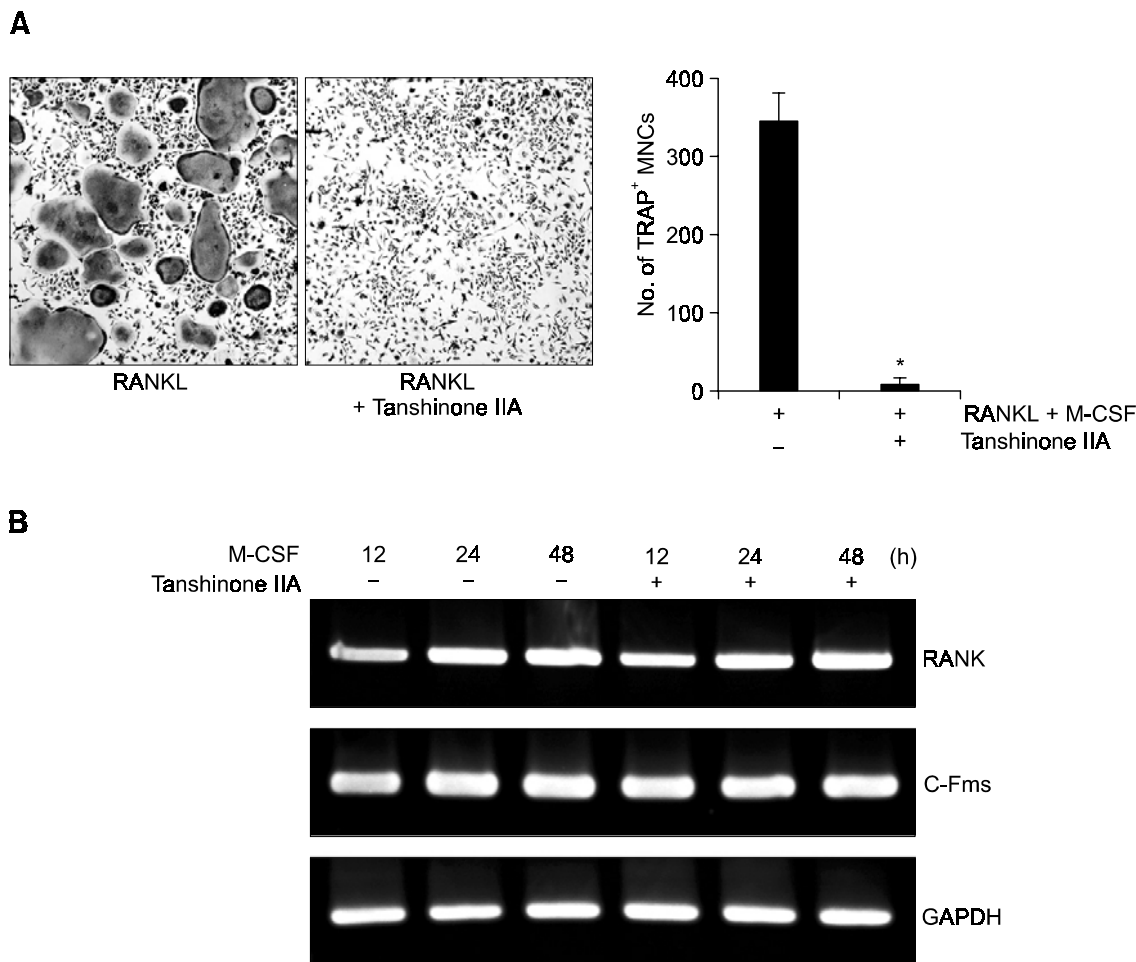

Figure 1. Inhibition of RANKL-mediated osteoclastogenesis by tanshinone IIA. (A) Osteoclast precursors were cultured with M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 4 days in the presence or absence of tanshinone IIA. Cells were fixed in 10% formalin, permeabilized with 0.1% Triton X-100, and stained with TRAP solution. (B) TRAP$^+$ cells containing five or more nuclei were counted as multinucleated osteoclasts. Data from three experiments are presented as mean ± S.D. *$P < 0.01$, significantly different from the control. (C) Osteoclast precursors were incubated with M-CSF (30 ng/ml) in the presence or absence of tanshinone IIA for the indicated time. The mRNA expression of the indicated genes was analyzed by RT-PCR.

Statistical analysis

All quantitative results are presented as means $\pm$ S.D. Statistical significance was analyzed by using Student's *t* test. *P* values < 0.05 were considered to be statistically significant and are defined with an asterisk.

Results

Tanshinone IIA inhibits RANKL-mediated osteoclast differentiation

Tanshinone IIA greatly suppressed RANKL-mediated osteoclast differentiation in osteoclast precursors (Figure 1A and B). We also examined the alteration of expression of RANK and c-Fms, which act as receptors for RANKL and M-CSF, respectively. Osteoclast precursors were treated with DMSO or tanshinone IIA for various times in the presence of M-CSF. M-CSF increased RANK mRNA expression in osteoclast precursors, as previously reported (Mi-

yamoto *et al.*, 2000). Tanshinone IIA did not change the expression of RANK and c-Fms in osteoclast precursors (Figure 1C). These results suggest that tanshinone IIA does not inhibit signaling pathways, which involved in M-CSF-induced RANK expression and that tanshinone IIA may affect RANKL-induced signaling involved in osteoclastogenesis.

Tanshinone IIA suppresses c-Fos and NFATc1 expression induced by RANKL

Osteoclast differentiation is regulated through the induction of various genes in response to RANKL. In particular, c-Fos and NFATc1 are well known to play an essential role in the differentiation of osteoclast precursors (Takayanagi *et al.*, 2002; Hirotani *et al.*, 2004). We thus studied whether tanshinone IIA regulates the expression of c-Fos and NFATc1 in response to RANKL. Osteoclast precursors were pretreated with DMSO or tanshinone IIA (10 μg/ml) and further stimulated with RANKL (100 ng/ml) for various times in the presence of M-CSF. Densito-
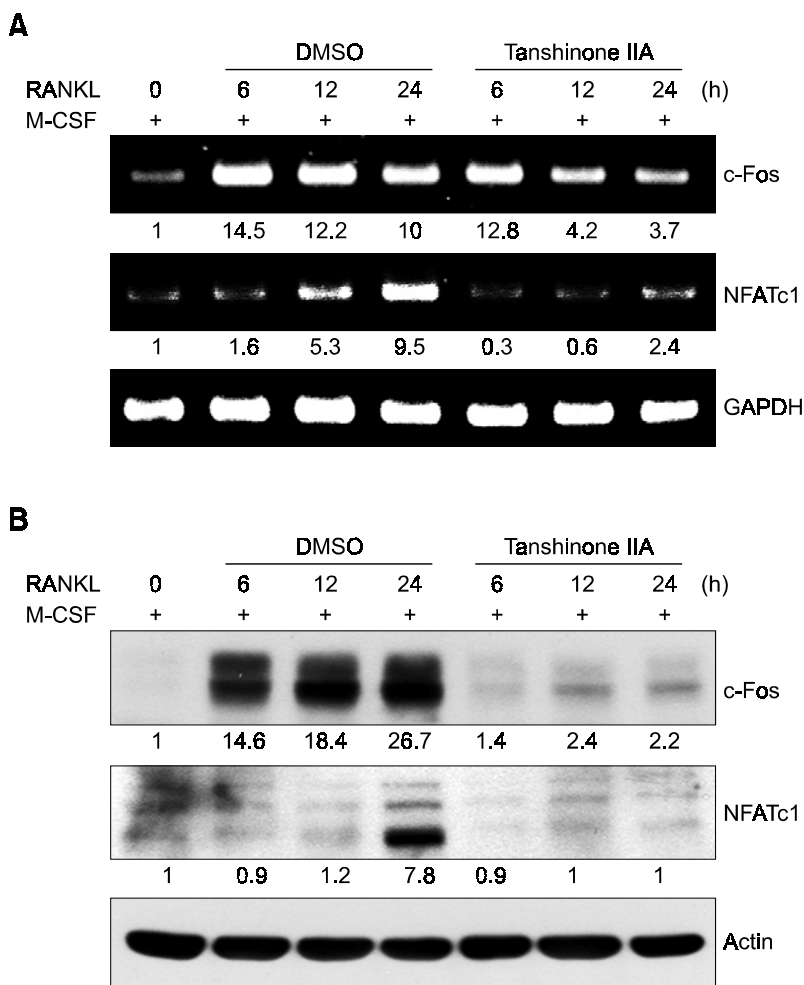

Figure 2. Inhibition of RANKL-induced c-Fos and NFAT2 expression in osteoclast precursors by tanshinone IIA. (A) Osteoclast precursors were pretreated for 1 h with or without tanshinone IIA and then stimulated with RANKL (100 ng/ml) in the presence of M-CSF (30 ng/ml) for the indicated time. Total RNA was isolated and reverse transcribed, and the mRNA expression of the indicated genes was measured by RT-PCR. (B) Cells were stimulated as in (A). Cells lysates were immunoblotted with antibodies to p38 and NFATc1 to detect c-Fos and NFATc1. Band intensity was quantified by using an Image Pro-plus program version 4.0 (Media Cybernetics).
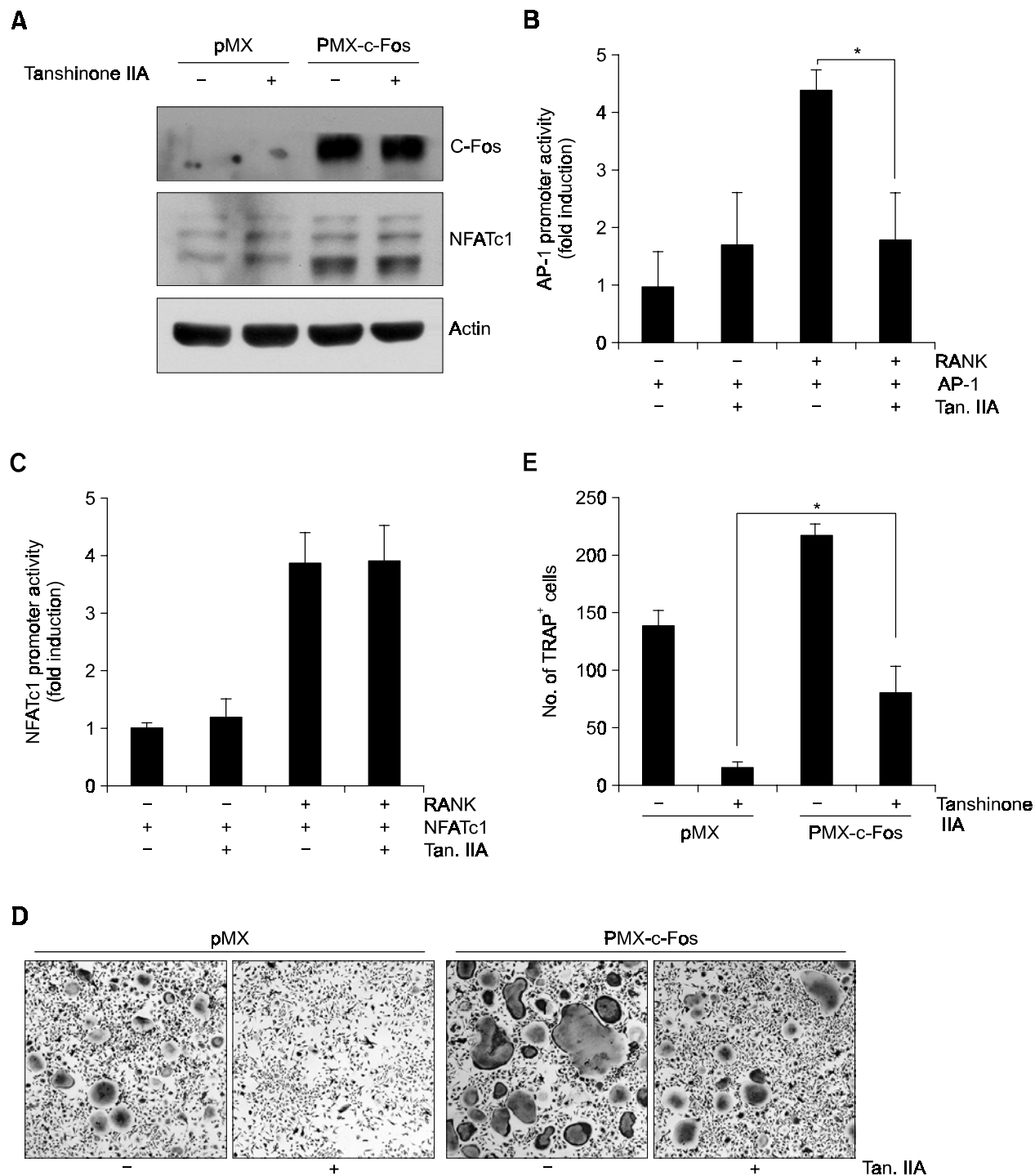
Figure 3. Overexpression of c-Fos reverses the inhibitory effect of tanshinone IIA on osteoclast differentiation. pMX-IRES-EGFP or pMX-c-Fos-IRES-EGFP vectors were transiently transfected into Plat E cells. After 6-8 h, the medium was changed and the culture supernatant fluid was used as a retrovirus as described in Materials and methods. (A) Osteoclast precursors were infected with control or c-Fos retrovirus for 7 h in the presence of M-CSF (30 ng/ml) and polybrene (6 μg/ml). The medium was replaced and further treated with or without tanshinone IIA (10 μg/ml) for 48 h. Cell lysates were immunoblotted with antibodies to c-Fos and NFATc1. (B) HEK 293T cells were seeded in a 24-well plates and transiently transfected with AP-1 reporter gene or RANK and AP-1 reporter gene. Cells were incubated for 24 h in the presence or absence of tanshinone IIA. 24 h after transfection, the cells were lysed and assayed for luciferase activity using a luciferase reporter assay system. (C) HEK 293T cells were transiently transfected with NFATc1 reporter gene or RANK and NFATc1 reporter gene. Luciferase assay was performed as above. (D) Osteoclast precursors were infected with control or c-Fos retrovirus as in (A) and were then cultured for 5 days with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the presence or absence of tanshinone IIA (10 μg/ml). Cells were fixed and stained for TRAP. (E) TRAP+ cells were counted as osteoclasts.

metric value of RT-PCR normalized by GAPDH intensity showed that tanshinone IIA greatly inhibited the expression of NFATc1 mRNA and slightly suppressed c-Fos mRNA expression in response to RANKL (Figure 2A). Consistent with the results of the RT-PCR analyses, protein levels of c-Fos and NFATc1 were increased in response to RANKL, but the expression of both c-Fos and NFATc1 was significantly inhibited by tanshinone IIA (Figure 2B). Thus, tanshinone IIA may inhibit osteoclast differentiation by inhibiting c-Fos and NFATc1 expression in response to RANKL.

Inhibition of osteoclast differentiation by tanshinone IIA is reversed by overexpression of c-Fos

To elucidate the mechanism by which tanshinone IIA inhibits c-Fos and NFATc1 expression, we infected osteoclast precursors with control retrovirus or c-Fos retrovirus for 7 h in the presence of M-CSF (30 ng/ml) and polybrene (6 μg/ml). The medium was replaced with fresh α-MEM containing 10% FBS and antibiotics and further cultured for 48 h with M-CSF in the presence or absence of tanshinone IIA. As shown in Figure 3A, the expression of c-Fos and NFATc1 induced by c-Fos retroviral infection com-
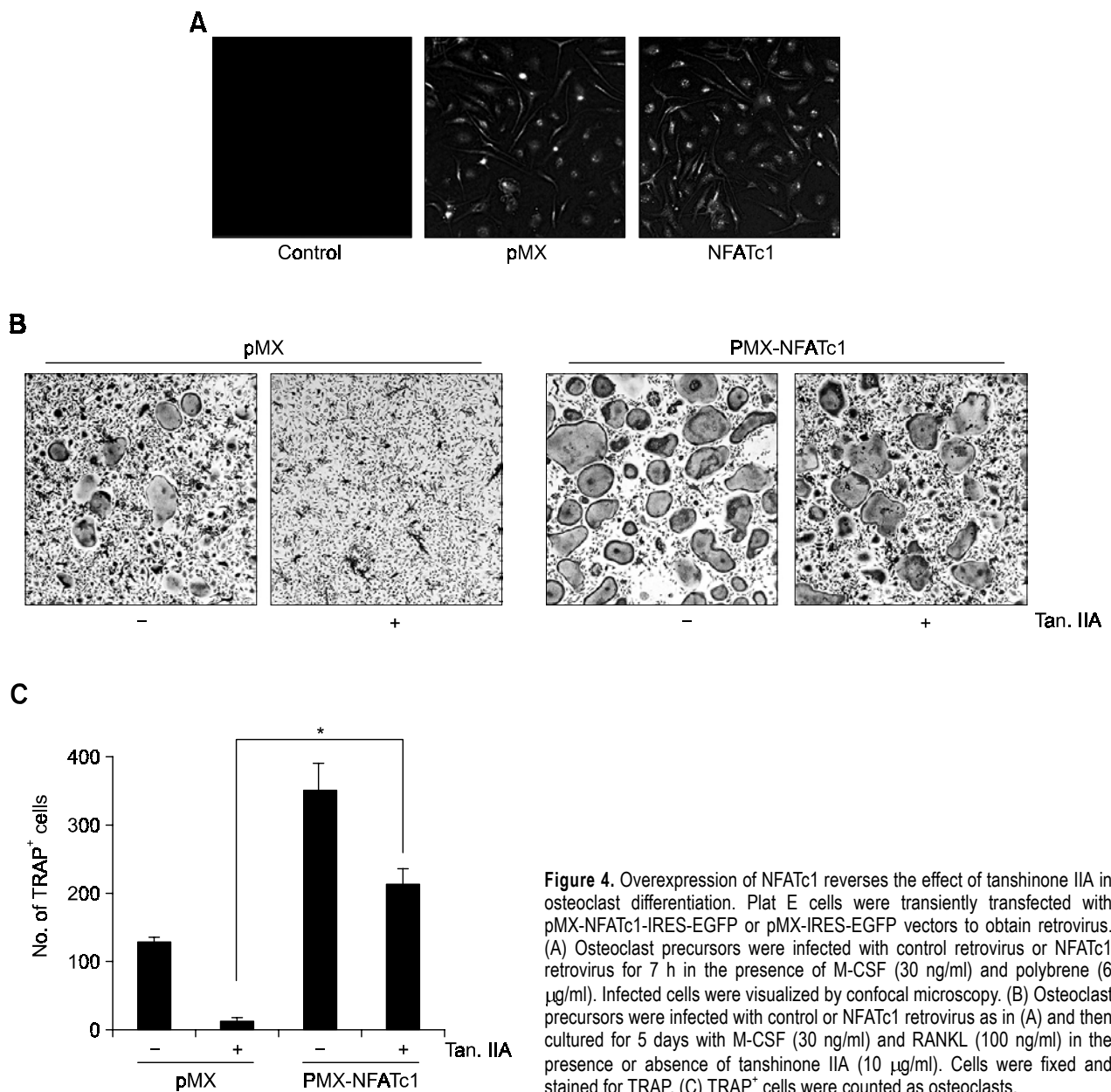

Figure 4. Overexpression of NFATc1 reverses the effect of tanshinone IIA in osteoclast differentiation. Plat E cells were transiently transfected with pMX-NFATc1-IRES-EGFP or pMX-IRES-EGFP vectors to obtain retrovirus. (A) Osteoclast precursors were infected with control retrovirus or NFATc1 retrovirus for 7 h in the presence of M-CSF (30 ng/ml) and polybrene (6 μg/ml). Infected cells were visualized by confocal microscopy. (B) Osteoclast precursors were infected with control or NFATc1 retrovirus as in (A) and then cultured for 5 days with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the presence or absence of tanshinone IIA (10 μg/ml). Cells were fixed and stained for TRAP. (C) TRAP$^+$ cells were counted as osteoclasts.

pletely rescued the inhibitory effect of tanshinone IIA. Furthermore, we examined for direct evidence that tanshinone IIA inhibits the c-Fos and NFATc1 transcriptional activity by cotransfecting HEK 293T cells with RANK and the AP-1 or NFATc1 promoter-luciferase gene. RANK-mediated AP-1 promoter activity was inhibited in the presence of tanshinone IIA. However, tanshinone IIA does not inhibit NFATc1 transcriptional activity (Figure 3B and C). These results suggest that tanshinone IIA specifically regulates c-Fos expression in response to RANKL rather than regulating NFATc1 expression.

Next we investigated whether overexpression of c-Fos in osteoclast precursors could rescue the inhibition of osteoclast differentiation by tanshinone IIA. After infection with control or c-Fos retrovirus, cells were cultured for 5 days with M-CSF and RANKL in the presence or absence of tanshinone IIA. Osteoclast differentiation was significantly higher after infection of osteoclast precursors with c-Fos retrovirus than after infection with control retrovirus in the presence of tanshinone IIA (Figure 3D and E). These results suggest that tanshinone IIA suppresses osteoclast differentiation through inhibition of c-Fos expression.

NFATc1 rescues osteoclast differentiation in the presence of tanshinone IIA

The induction of NFATc1 in osteoclast precursors is essential for osteoclast differentiation and is regulated by both c-Fos and the calcium/calcineurin pathways (Takayanagi *et al.*, 2002). Inhibition of NFATc1 by tanshinone IIA is associated with inhibition of c-Fos in response to RANKL. Therefore, we examined whether NFATc1 could overcome the inhibition of osteoclast differentiation caused by tanshinone IIA. After infection of osteoclast precursors with control and NFATc1 retrovirus, the medium was replaced with fresh α-MEM containing 10% FBS and antibiotics and further cultured for 48 h with M-CSF. GFP-expressed cells were visualized by confocal microscopy, and the infection efficiency of the control and NFATc1 retroviruses was more than 90% (Figure 4A). Osteoclast precursors were infected with control or NFATc1 retrovirus in the presence of M-CSF (30 ng/ml) and polybrene (6 μg/ml) and further cultured for 5 days with M-CSF (30 ng/ml) and RANKL (50 ng/ml) in the presence or absence of tanshinone IIA (10 μg/ml). As shown in Figure 4B and C, overexpression of NFATc1 rescued the blockage of osteoclast differentiation in the presence of tanshinone IIA. Thus, the inhibitory effects of tanshinone IIA on osteoclast differentiation result from the decrease in NFATc1 by inhibition of c-Fos.

Discussion

Bone is a dynamic tissue that is maintained by the balance between bone formation and bone resorption; this balance can be easily upset by chronic inflammation, menopause, and aging (Karsenty *et al.*, 2002). Bone erosion results from excessive osteoclast formation rather than damage to osteoblast activity. Therefore, the development of drugs that act to regulate osteoclast formation is important to prevent bone disease. Previously, we reported that tanshinone IIA significantly inhibits the differentiation of osteoclast precursors mediated by RANKL (Kim *et al.*, 2004). Two key factors, RANKL and M-CSF, regulate the expression of various genes in osteoclast precursors by binding to their receptors, RANK and c-Fms, respectively, the levels of which are important in osteoclast differentiation. In particular, expression of c-Fms is a crucial feature of osteoclast progenitors. The addition of M-CSF to osteoclast progenitors causes the expression of RANK; thereby, RANKL induces differentiation into osteoclasts (Miyamoto *et al.*, 2000). Although tanshinone IIA inhibits osteoclast differentiation, it does not alter the expression of RANK or c-Fms (Figure 1). These results suggest that tanshinone IIA suppresses osteoclast differentiation induced by RANKL.

A member of the TNF superfamily, RANKL mediates multiple signaling pathways via RANK in osteoclast precursors and activates critical transcription factors for osteoclast differentiation and function (Suda *et al.*, 1999; Boyle *et al.*, 2003; Lee and Kim, 2003). One such transcription factor is NFATc1, which is expressed in osteoclast precursors through Ca^{2+} oscillation and c-Fos in response to RANKL and that is required for the regulated expression of many genes, including TRAP, calcitonin receptor, and cathepsin K (Takayanagi *et al.*, 2005). Thus, regulation of NFATc1 expression in osteoclast precursors has significant consequences for bone disorder therapeutics. We found that the inhibition of osteoclast differentiation by tanshinone IIA coincides with regulation of c-Fos and NFATc1. Tanshinone IIA slightly inhibits the mRNA expression of endogenous c-Fos induced by RANKL but completely inhibits the expression of the c-Fos protein (Figure 2). Therefore, we hypothesized that down-regulation of c-Fos protein is involved in c-Fos mRNA degradation. However, tanshinone IIA did not affect the expression of exogenous c-Fos induced by c-Fos retroviral infection (Figure 3) or the stability of c-Fos mRNA (data not shown). These results indicate that tanshinone IIA does not affect the mRNA or protein stability of c-Fos or its posttranscriptional regulation. Our previous results indicate that tanshinone IIA

causes alteration of RANKL-induced signaling pathways including ERK (Kim *et al.*, 2004), a known signaling pathway to regulate c-Fos expression (Whitmarsh *et al.*, 1995). Thus, this altered signaling pathway is related to inhibitory effect of tanshinone IIA on RANKL-induced c-Fos expression.

c-Fos, however, plays an important role in the expression of NFATc1, which is an essential factor for osteoclast differentiation. We also found that overexpression of c-Fos caused by c-Fos retroviral infection can rescue NFATc1 expression in the presence of tanshinone IIA (Figure 3). However, the inhibitory effect of tanshinone IIA on RANKL-mediated osteoclast differentiation was not entirely rescued by c-Fos or NFATc1 administration. Actually, NFATc1 is essential for osteoclast differentiation but is not sufficient for osteoclast differentiation in the absence of RANKL. Other transcription factors, including NF-κB, MITF, activator protein 1, and PU1, are required for osteoclast differentiation (Teitelbaum *et al.*, 2003; Walsh *et al.*, 2003). These results indicate that osteoclast differentiation induced by RANKL requires several transcription factors for cooperating with NFATc1. We showed previously that tanshinone IIA suppresses Akt, ERK, and NF-κB activation in response to RANKL (Kim *et al.*, 2004). Thus, we conclude that tanshinone IIA prevents functional cooperation between NFATc1 and NF-κB in response to RANKL.

In summary, we investigated the effect of tanshinone IIA on osteoclast formation. Tanshinone IIA greatly inhibits osteoclast formation by inhibiting the expression of c-Fos and NFATc1 induced by RANKL. Taken together, our results suggest that tanshinone IIA probably plays an important role in inhibiting bone loss by preventing osteoclast formation.

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