# Radix Dipsaci total saponins stimulate MC3T3-E1 cell differentiation via the bone morphogenetic protein-2/MAPK/Smad-dependent Runx2 pathway

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**Abstract.** Radix Dipsaci total saponins (RTS) are primary active components of Radix Dipsaci, which is administered orally for the treatment of osteoporosis according to Chinese Medicine. RTS have also been shown to reduce the risk of bone fractures in rats. However, the detailed molecular mechanisms underlying their action remain elusive. In the present study, the ability of RTS to increase alkaline phosphatase activity, osteocalcin levels and the degree of mineralization was investigated in MC3T3-E1 mouse osteoblast precursor cells. In addition, the associated molecular mechanism was detected. The results revealed that RTS exerted an effect on osteoblastic maturation and differentiation. Induction of differentiation by RTS was associated with an increase in the expression levels of bone morphogenetic protein-2 (BMP-2), phosphorylated (P)-Smad1/5/8, P-ERK1/2, P-p38 and Runt-related transcription factor 2 (Runx2). Blocking BMP-2 expression with noggin significantly reduced the levels of osteoblastic differentiation and subsequently attenuated the expression levels of P-Smad1/5/8, P-ERK1/2, P-p38 and Runx2. This indicated that RTS induced osteoblastic differentiation through BMP-2/mitogen-activated protein kinase/Smad1/5/8-dependent Runx2 signaling pathways and that it may be a promising agent for enhancing bone formation.

## Introduction

The survival of osteoblasts is one of the determinants of the development of osteoporosis. The drugs currently used in the treatment of osteoporosis are bone resorption inhibitors, including bisphosphonates, calcitonin and estrogen (1,2). However, the positive effect of these drugs on the recovery of bone mass is moderate (1). Therefore, satisfactory anabolic agents are urgently required. Through increasing the proliferation of an osteoblastic lineage, or inducing differentiation and mineralization, these agents stimulate an increase in bone tissue and the prevention of bone destruction (3,4).

As mediators in the cell signaling pathways associated with bone formation, bone morphogenetic proteins (BMPs) have an important role in the differentiation of osteoblasts (5-7). The Smad and mitogen-activated protein kinase (MAPK) pathways are essential components of BMP signaling during osteoblast differentiation (8-10). Among the members of the BMP subfamily, BMP-2 is able to induce bone formation and differentiation *in vivo* and *in vitro* (11,12). BMP-2 activates ERK1/2, p38, c-Jun kinases and Smad1/5/8 proteins (13-16), and induces the expression of core binding factor (Cbfa)1/Runt-related transcription factor 2 (Runx2) (17,18), an important transcription factor in osteoblastic differentiation (19).

A previous study by our group demonstrated that RTS effectively inhibited osteoporosis in ovariectomized rats. Additionally, RTS has been shown to enhance MC3T3-E1 cell differentiation, potentially due to its role in increasing the expression levels of BMP-2 (20). However, the detailed molecular mechanisms of the osteogenic effects of RTS remain to be determined. In the present study, the effects of RTS on the osteogenic activities of MC3T3-E1 cells were investigated. In addition, in order to establish the potential mechanisms involved in the osteoprotective effects of RTS, the levels of BMP-2, Smad1/5/8, MAPKs and Runx2 were assayed, proteins which are associated with the osteogenesis signaling pathways.

## Materials and methods

*Cells and reagents.* MC3T3-E1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in α-minimum essential medium (α-MEM;

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HyClone Laboratories, Inc., Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc.) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% $CO_2$ and 95% air at 37˚C. Radix Dipsaci was purchased in a local Chinese Medicine store and was identified morphologically, histologically and chemically using standard Chinese Pharmacopoeia procedures (Chinese Pharmacopeia Commission, 2010). In brief, RTS was isolated and purified by refluxing in 60% ethanol and D101 macroporous resin, repectively. RTS was analyzed by colorimetric determination using asperosaponin VI (Push Bio-Technology Co. Ltd , Chengdu, China) as the standard and the content of RTS was 76.5% (23).

*Alkaline phosphatase (ALP) activity assay*. Osteoblasts were seeded at a density of $2x10^4$ cells/well and cultured in 24-well plates with α-MEM containing 10% FBS, L-ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) in the presence or absence of RTS (30, 100 or 300 μg/ml). The cells were washed twice with phosphate-buffered saline (PBS; Wuhan Boster Biological Technology, Inc., Wuhan, China), lysed with 0.2% Triton X-100 (Tianjin Damao Chemical Reagent Factory, Tianjin, China) and the lysate was centrifuged at 14,000 x g for 5 min. The supernatant was collected in order to measure the ALP activity and protein concentration using an ALP activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Chian) and a BCA protein assay kit (Beyotime, Shanghai, China), respectively (21).

*Assessment of osteocalcin*. Osteocalcin ELISA kits (Elabscience Biotechnology Co., Ltd, Wuhan, China) were used to detect osteocalcin levels. Cells were treated with RTS (30, 100 or 300 μg/ml) for eight days. The samples were placed in 96-well microtiter plates coated with mouse monoclonal detective antibodies for osteocalcin from the kit and incubated for 2 h at room temperature according to the manufacturer's instructions. Following removal of the unbound material using washing buffer, horseradish peroxidase (HP)-conjugated streptavidin was added to bind to the antibodies. HP catalyzes the conversion of the chromogenic tetramethylbenzidine to a colored solution, with the color intensity in proportion to the amount of protein present in the sample. The absorbance of each well was measured at a wavelength of 450 nm (Synergy™ HT Multi-Mode Microplate Reader; Bio-Tek Instruments, Inc., Winooski, VT, USA). Results are presented as the percentage change in the activity of the treated cells compared with that of the untreated control.

*Mineralized matrix assay*. Mineralization was determined via staining with Alizarin Red-S (Aldrich, Milwaukee, WI, USA). MC3T3-E1 cells were seeded into 12-well plates at a density of $2x10^5$ cells/well. Following two days of incubation, cells were washed twice with PBS solution, treated with or without RTS and cultured in α-MEM containing 10% FBS, L-ascorbic acid (50 μg/ml; Sigma-Aldrich) and β-glycerophosphate (10 mM; Sigma-Aldrich). During this period, the medium was changed every three days. Following a 14-day incubation with or without drugs, the cells were fixed with 70% ethanol for 1 h, washed three times with distilled water and then incubated with 40 mmol/l Alizarin Red-S (pH 4.2) for 10 min at 37˚C. Once stained, the cultures were washed three times with deionized water and then incubated with PBS for a further 15 min. Images of the mineralized matrices were captured using a microscope (80i; Nikon, Tokyo, Japan). To quantify the matrix mineralization, Alizarin Red-S-stained cultures were incubated in 100 mmol/l cetylpyridinium chloride (Tianjin Damao Chemical Reagent Factory) for 1 h in order to solubilize and release calcium-bound Alizarin Red-S into the solution. The absorbance of the released Alizarin Red-S (Sigma-Aldrich) was measured at a wavelength of 570 nm (Synergy™ HT Multi-Mode Microplate Reader; Bio-Tek) (24).

*Western blot analysis*. To detect protein expression following RTS treatment, MC3T3-E1 cells were lysed and the cell lysates were harvested and maintained on ice for 30 min. Once the soluble fractions of nuclear and cytoplasmic proteins were obtained they were used for western blotting. Equal amounts of protein were subjected to 15% SDS-PAGE (Wuhan Boster Biological Technology, Inc.). The proteins were transferred to nitrocellulose membranes (Pall, Port Washington, NY, USA) using transfer buffer (50 mM Tris, 190 mM glycin and 10% methanol; Tianjin Damao Chemical Reagent Factory) at 50 V for 2.5 h. The membranes were incubated with blocking buffer containing 0.05% Tween-20 (Tianjin Damao Chemical Reagent Factory) and 5% non-fat milk for 12 h at 4˚C. Following washing three times with PBS, the blot was incubated with primary antibodies (rabbit polyclonal anti-BMP-2, 1:200; rabbit polyclonal anti-Smad1/5/8, 1:200; rabbit polyclonal anti-phosphorylated (P)-Smad1/5/8, 1:200; rabbit polyclonal anti-p38, 1:200; rabbit polyclonal anti-P-p38, 1:200; rabbit polyclonal anti-ERK1/2, 1:200; rabbit polyclonal anti-P-ERK1/2, 1:200; rabbit polyclonal anti-JNK, 1:200; rabbit polyclonal anti-P-JNK, 1:200; rabbit polyclonal anti-Runx2, 1:200; rabbit polyclonal anti-histone, 1:500; and mouse monoclonal anti-β-actin, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 12 h at 4˚C. Subsequently, the membranes were washed three times for 10 min with tris-buffered saline (TBS) buffer containing 0.05% Tween-20 and subsequently incubated with anti-rabbit or anti-mouse immunoglobulin G secondary antibodies (1:5,000 dilution) for 1 h at room temperature. The membranes were washed three times for 10 min with Tris-buffered saline (Tianjin Damao Chemical Reagent Factory) and once for 10 min with PBS. Following reaction and coloration using enhanced chemiluminescence (Millipore, Billerica, MA, USA), the relative values for the absorbance of the bands and the absorbance of β-actin or histone were compared.

*Statistical analysis*. Data were analyzed using a one-way analysis of variance test to compare the different groups. Results are presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*RTS enhances the differentiation of MC3T3-E1 cells*. As shown in Fig. 1A, RTS treatment increased the level of ALP

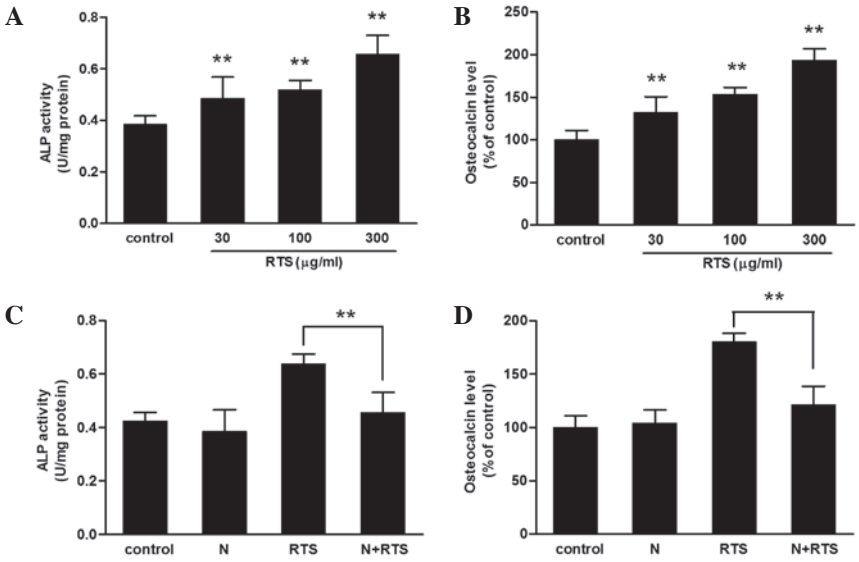


Figure 1. Enhancement of MC3T3-E1 cell differentiation by RTS. RTS increased (A) ALP activity and (B) osteocalcin production. Noggin inhibited the upregulation of (C) ALP and (D) osteocalcin production by RTS. ALP activity was assessed using a commercial ALP kit. The amount of osteocalcin in the culture medium was assessed using osteocalcin ELISA kits. Values are presented as the mean ± standard deviation (n=6). **P<0.01 vs. control. RTS, Radix Dipsaci total saponins; ALP, alkaline phosphatase.

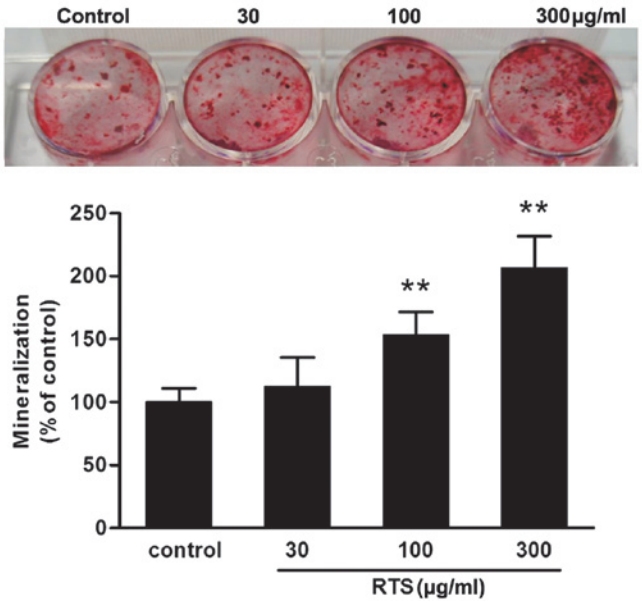


Figure 2. RTS increased the mineralization of MC3T3-E1 cells. The mineralized nodule formation was assessed by Alizarin Red-S staining. The bound stain was washed with a solution of 100 mmol/l cetylpyridinium chloride and quantified using a Bio-Rad ELISA reader. Values are presented as the mean ± standard deviation (n=3). **P<0.01 vs. control. RTS, Radix Dipsaci total saponins.

activity in a concentration-dependent manner. Additionally, the expression levels of osteocalcin protein were increased by RTS in a concentration-dependent manner in following 7 days of treatment (Fig. 1B); the levels in the 30, 100 and 300 $\mu$g/ml RTS-treated groups were significantly higher than those in the controls (P<0.01).

*RTS promotes mineralization of MC3T3-E1 cells*. The calcified nodules stained positive with Alizarin Red-S (Fig. 2). The 100 and 300 $\mu$g/ml RTS-treated groups had the highest number of mineralized nodules (P<0.01 compared with the control group).

*RTS induces the differentiation of MC3T3-E1 cells via the BMP-2 pathway*. As shown in Fig. 3A, BMP-2 protein levels were markedly increased by RTS treatment in a concentration-dependent manner. MC3T3-E1 cells were pretreated with noggin protein (100 ng/ml) for 2 h, then 300 $\mu$g/ml RTS was added for 24 h. RTS-induced BMP-2 protein expression was diminished by the concurrent treatment with noggin (Fig. 3B). In addition, the RTS-induced ALP activity and osteocalcin protein levels were significantly diminished in cells treated concurrently with noggin compared with those in untreated cells (Fig. 1C and D). Therefore, RTS-mediated cell differentiation may proceed via a BMP-2-dependent pathway.

*BMP-2 is involved in the activation of Smad1/5/8, ERK and p38 in RTS-treated cells*. Binding of BMP-2 to the BMP receptor activates MAPKs or SMADs via phosphorylation (15,16). RTS treatment significantly increased the expression of P-Smad1/5/8, P-p38 and P-ERK1/2 (Fig. 3A). The activation of Smad1/5/8, p38 and ERK1/2 was blocked in MC3T3-E1 cells pretreated with 100 ng/ml noggin protein for 2 h and then co-treated with 300 $\mu$g/ml RTS for 24 h (Fig. 3B).

*BMP-2 is required to increase expression of Runx2 in RTS-treated MC3T3-E1 cells*. Runx2 is a vital transcription factor required for osteoblast differentiation (19). Following treatment with RTS for 24 h, the expression levels of Runx2 protein in cells were markedly increased (Fig. 4A). When cells were incubated with RTS in the presence of noggin, the stimulatory effect of RTS on the expression of Runx2 protein were markedly reduced (Fig. 4B). These results indicated that RTS activated osteogenic differentiation through the BMP-2/MAPK/Smad-dependent Runx2 signaling pathway.

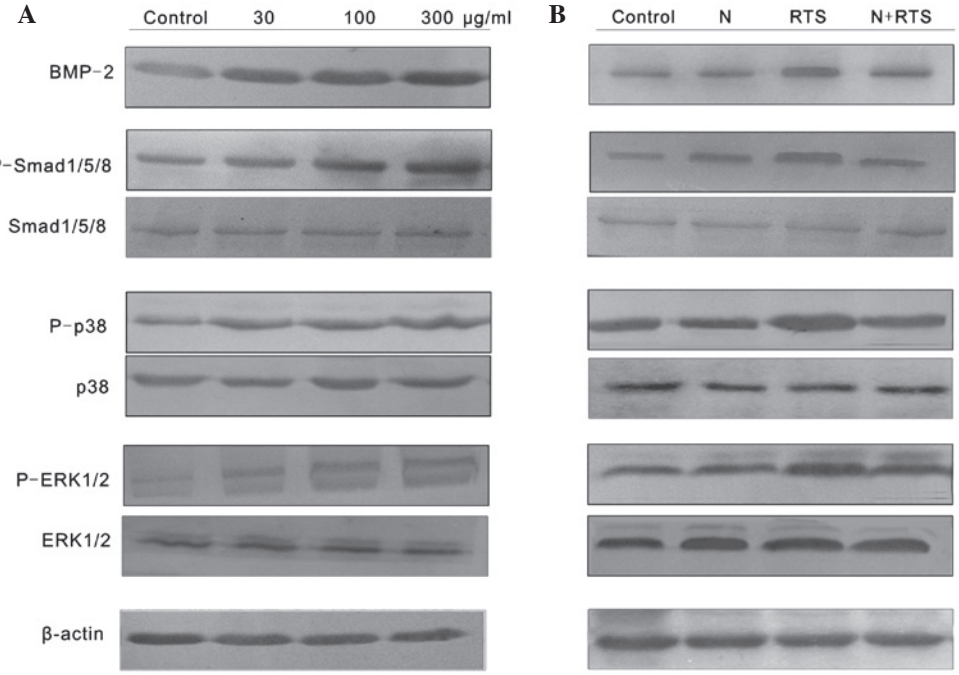


Figure 3. Western blots showing the protein levels of BMP-2, Smad1/5/8, p38 and ERK1/2 in MC3T3-E1 cells. (A) Protein in cells treated with RTS; (B) Noggin inhibited the induction of BMP-2, P-Smad1/5/8, P-p38 and P-ERK1/2 by RTS. Blots are representatives of three independent experiments. N, noggin; RTS, Radix Dipsaci total saponins; P, phosphorylated protein; BMP, bone morphogenetic protein.

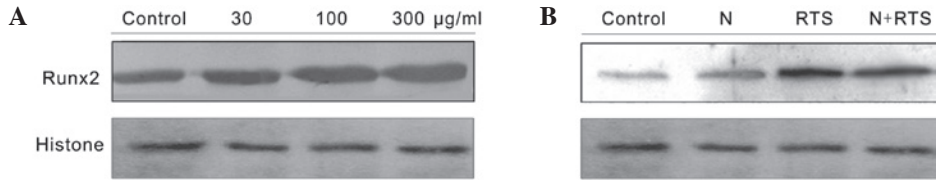


Figure 4. Western blots showing the protein levels of Runx2 in MC3T3-E1 cells. (A) Runx2 levels following treatment with RTS; (B) Noggin inhibited the induction of Runx2 by RTS. Blots are representatives of three independent experiments. N, noggin; RTS, Radix Dipsaci total saponins.

## Discussion

The anti-osteoporosis effect of RTS *in vivo* was systemically evaluated in a previous study by our group (20). In the present study, the effects of RTS on the osteogenic activities in MC3T3-E1 cells and the potential mechanisms of action were evaluated. Treatment of MC3T3-E1 cells with RTS not only raised the activity of ALP (a marker of maturation) but also increased the levels of osteocalcin proteins (late stage markers of differentiation). This indicated that RTS may affect cell differentiation processes from the early to terminal stages. In addition, RTS increased the mineralization (a marker of bone formation) of MC3T3-E1 cells.

BMPs have potent osteogenic effects and control osteoblast differentiation during osteogenesis. BMP-2, one of the BMP subfamilies, promotes differentiation through enhanced intracellular ALP activity as well as osteocalcin and collagen protein synthesis (23). The effect of BMPs is mediated by heterotetrameric serine/threonine kinase receptors and the downstream transcription factors Smad1/5/8. Upon phosphorylation by type I receptors, Smad1/5/8 form complexes with Smad4, translocate to the nucleus and regulate the transcription of target genes associated with differentiation (13). Several natural or chemical compounds, including daidzein, osthole and syringetin, have been reported to induce osteoblast differentiation by induction of BMP and/or SMAD signaling (24-26). The results of the present study showed that the expression levels of BMP-2 were enhanced and the phosphorylation of SMAD1/5/8 was significantly increased in RTS-treated MC3T3-E1 cells. RTS-mediated SMAD1/5/8 activation was blocked by the BMP antagonist noggin and cell differentiation was attenuated in MC3T3-E1 cells. Hence, the BMP-2 signaling system has an important role in RTS-mediated cell maturation and differentiation in MC3T3-E1 cells.

In addition to Smad activation, BMP-2 can activate Smad-independent pathways, for example the MAPK signaling pathway. BMP-2 can stimulate two MAPKs: ERK and p38. The activation of p38 and ERK is essential in the BMP-2-induced upregulation of AP, type I collagen, osteocalcin and osteopontin (27-29). The results of the present study showed an increase in p38 and ERK activity in RTS-treated cells. This suggested that the activation of p38 and ERK may have an important role in increasing BMP-2 levels and the cell differentiation in MC3T3-E1 cells stimulated by RTS. The increase in the expression of Runx2 protein by RTS is prevented by the BMP inhibitor noggin,

which demonstrates an involvement of the BMP-2 pathway in the stimulatory effect of RTS on Runx2.

In conclusion, the present study clearly demonstrated that RTS stimulates osteoblast differentiation at various stages in MC3T3-E1 cells. The effect of RTS on cell maturation and differentiation is strongly associated with the BMP-2/MAPK/Smad1/5/8-dependent Runx2 signaling pathway. This suggests that RTS may be beneficial in stimulating osteoblastic activity resulting in bone formation.

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