Astragaloside II induces osteogenic activities of osteoblasts through the bone morphogenetic protein-2/MAPK and Smad1/5/8 pathways

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Received December 26, 2011; Accepted February 20, 2012

DOI: 10.3892/ijmm.2012.941

Abstract. Radix Astragalus has been identified to exert beneficial effects in preventing postmenopausal bone loss. However, the active ingredients and mechanism of action remain unknown. In this study, we examined the effect of Astragaloside II (AST II), which is a monomer of Astragalus saponin, on the viability, proliferation, differentiation and maturation of rat primary osteoblasts, as well as its relevant molecular mechanism. We found that AST II exhibits a significant induction of proliferation, differentiation and mineralization in primary osteoblasts. AST II stimulates osteoblast differentiation at various stages, from early to late stage of differentiated osteoblasts. Furthermore, induction of differentiation by AST II is associated with increased expression of bone morphogenetic protein-2 (BMP-2), activation of Smad1/5/8, ERK1/2 and p38, and increased expression of core-binding factor 1 (Cbfa1)/Runx2. BMP antagonist (Noggin) blocks the effect of AST II on cell differentiation, and Smad1/5/8, p38, Cbfa1 expression, but only partly decreases ERK1/2 activation. This indicates that BMP-2 is essential in AST II-mediated osteoblast differentiation and Smad1/5/8, p38, Cbfa1 activation, and is partly involved in ERK1/2 activation. In conclusion, although *in vivo* studies are required in the future, as a phyto-saponin of Radix Astragalus, AST II may become a novel candidate that is beneficial for stimulating the osteoblastic activity resulting in bone formation, which has not been recognized and reported previously.

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Key words: Astragaloside II, osteoblasts, osteogenic activities, bone morphogenetic protein-2, Smad1/5/8, mitogen-activated protein kinases

Introduction

Osteoporosis is a reduction in skeletal mass due to an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts. The formation of bone involves a complex series of events, including the proliferation and differentiation of osteoblasts and result eventually in the formation of a mineralized extracellular matrix (1,2). Among cell signaling pathways associated with bone formation, bone morphogenetic proteins (BMPs) play important roles in bone formation and osteoblasts differentiation by stimulating alkaline phosphatase (ALP) activity and synthesis of proteoglycan, collagen, fibronectin and osteocalcin (3-5). In the BMP subfamily, BMP-2 has demonstrated a strong osteo-inductive capacity *in vivo* and *in vitro* (6,7). It transduces signals through Smad-dependent and Smad-independent signalling pathways (8). BMP-2 stimulates the activation (phosphorylation) of Smad1/5/8 proteins (9,10) and extracellular signal-regulated protein (ERK)1/2, p38, c-Jun kinases mitogen-activated protein kinases (MAPKs) (9,11,12). Besides, the core binding factor (Cbfa1/Runx2) was reported as the most important transcription factor for osteoblastic differentiation (13). Both the Smad and MAPK pathways are essential components of the BMP signaling during osteoblast differentiation (11,14-16) and for Cbfa1 induction (17,18). Cbfa1 is also a pivotal target of some anti-osteoporosis drugs (17,19,20).

Patients or potential patients of osteoporosis need long-term treatment or prevention. Current drugs used to treat osteoporosis include bone resorption inhibitors, such as bisphosphonates, calcitonin, estrogen, and ipriflavone, and bone formation promoters, such as fluoride, PTH and PGE. However, what concerns people are side effects of the long-term use of these drugs. For instance, estrogen may cause a higher incidence of breast carcinoma, endometrial cancer, and cardiovascular disease (21). Besides, the high costs of some drugs are unacceptable for some osteoporosis patients, especially in developing countries. Considering the broad spectrum effect of osteoporosis in the medical system, and the defects of existing drugs, succedaneum such as traditional Chinese medicine may be a more acceptable and safer approach to

prevent osteoporosis. Plant-derived medicines display less adverse effects and have been a part of traditional healthcare in China for thousands of years. Many of these possess anti-osteoporotic activities (22,23).

The famous traditional Chinese Medicine Radix Astragalus is the root of *Astragalus membranaceus* (Fisch.) Bge. Radix Astragalus has been used as one of the superior 'tonics' in Chinese medicine for thousands of years, and used as an approved drug in the modern Pharmacopoeia of the People's Republic of China. It is noteworthy that Astragalus is one of the most frequently used herbs in formulas that are prescribed for the treatment of osteoporosis in China, such as Astragalus Sanxian Decoction (24), Drynol Cibotinis (25), and Buzhongyiqi Decoction (26), indicating the activity of anti-osteoporosis. Based on both the *in vitro* (27) and *in vivo* studies (28), Radix Astragalus were proved to be able to inhibit bone resorption, stimulate bone formation, and prevent ovariectomy-induced osteoporosis. However, as most of anti-osteoporosis studies on Astragalus were focused on the crude extract, the constituents active against anti-osteoporosis are still unknown. No systematic studies have so far been carried out to evaluate the effects of the active ingredients of Astragalus on the proliferation and differentiation of osteoblasts, and mechanism of the action.

Saponins have been reported to induce osteoblast osteogenesis. Researches have mainly focued on Panax notoginseng (29) and Panax ginseng (30) saponins. Astragalus saponins are the main active constituents of Astragalus. Radix Astragalus is mild, long-term use without significant side effects on the human body, and has been added in diet as a tonic in China for thousands of years. Moreover, compared with the rare and precious medicinal herbs (Panax notoginseng and Panax ginseng), Astragalus is much cheaper because of the rich plant resources and thus more affordable for patients. Therefore, we are extremely interested to investigate whether Astragalus saponins could be new anti-osteoporosis natural products or lead compounds. Astragaloside II (AST II) is a (3b, 6a, 16b, 20R, 24S)-3-((2-O-Acetyl-β-D-xylopyranosyl) oxy)-20, 24-epoxy-16, 25-dihydroxy-9, 19-cyclolanostan-6-yl β-D-glucopyranoside (Fig. 1). In the present study, we examined the concentration-dependent effects of AST II on the osteogenic activities, including proliferation, differentiation, as well as mineralization of osteoblasts. In addition, to establish the potential mechanism involved in the osteoprotective effects of AST II, we assayed BMP-2, SMADs, MAPKs and Cbfa1 levels, which are strongly associated with the signaling pathways of osteogenesis.

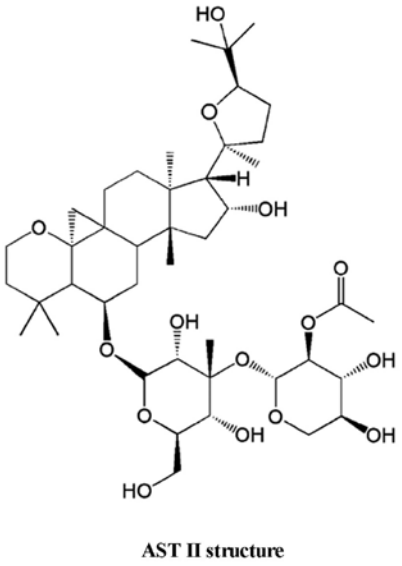

Figure 1. Structure of Astragaloside II (AST II).

Materials and methods

Reagents. AST II was purchased from Phytomarker Bio-Technology Co., Ltd. (China). Estradiol was purchased from Sigma (USA). Fetal bovine serum (FBS), minimal essential medium (MEM), were purchased from HyClone (USA). L-ascorbic acid, β-glycerophosphate and MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma. Ethynyl deoxyuridine (EdU) DNA imaging kit was purchased from RiboBio Co., Ltd. (China). ALP activity assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (China). Rat osteocalcin and collagen I ELISA kit was purchased from Biotech Co., Ltd. (China). Nuclear and cytoplasmic protein extraction kit and BCA-protein assay kit was purchased from Beyotime (Beyotime Institute of Biotechnology, Jiangsu, China). BMP-2 ELISA kit and noggin were purchased from R&D Systems (USA). The antibodies to Smad1/5/8, p38, ERK1/2, JNK, phosphorylated Smad1/5/8, phosphorylated p38, phosphorylated ERK1/2, phosphorylated JNK were purchased from Cell Signaling (USA), Cbfa1, Histone and β-actin from Santa Cruz Biotechnology, Inc. (USA). The stock solution of AST II was prepared at a concentration of 0.02 M of DMSO. It was then stored at -20˚C until use. For all experiments, the final concentrations of the test compound were prepared by diluting the stock with medium. Control cultures received the carrier solvent (0.05% DMSO).

Animals. Newborn Sprague-Dawley rats were obtained from the Experimental Animal Center, the Fourth Military Medical University. Animal care and experiments were approved and conducted in accordance with accepted standards of animal care and use as deemed appropriate by the Ethics Committee for Animal Care and Use of the Fourth Military Medical University.

Isolation and culture of rat calvarial osteoblasts. The calvaria of fetal rats were dissected from fetal rats. Primary osteoblastic cells were prepared by the method as previously described (31). The cells were cultured in MEM, supplemented with 10% FBS and antibiotics (100 U/ml of penicillin G and streptomycin 100 mg/ml) in a humidified atmosphere of 5% CO_2 and 95% air, at 37˚C. The characteristics of osteoblasts were confirmed by morphology, the expression of alkaline phosphatase, and the formation of mineralized matrix. All animals were treated according to the Guide for Care and Use of Laboratory Animals with the approval of Institutional Ethics Committee of the Fourth Military Medical University on animal experiment.

Cell viability assay. Cell viability was determined by means of MTT assay. Primary osteoblastic cells were seeded into 96-well plates at a density of $4x10^3$ cells/well in full medium

containing 10% FBS. After 24 h, the cells were washed twice with PBS solution and then AST II was added at concentrations ranging from 10^{-10} to 10^{-5} M in medium. Estradiol (E_2) at the concentration of 10^{-9} M was used as the positive control. After 48-72 h, 20 μl of MTT solution (0.5 mg/ml) were then added to each well and the mixture was incubated at 37˚C for 4 h to allow the formation of formazan crystal. Culture medium was then replaced with equal volume of DMSO to dissolve the dark blue crystals. After shaking at room temperature for 10 min to ensure that all crystals were totally dissolved, absorbance was measured at 490 nm by a Bio-Rad ELISA reader (Bio-Rad, CA, USA).

Cell proliferation assay. Cell proliferation was determined by means of EdU assay (32). Osteoblasts ($2x10^4$ cells/well) were seeded in 24-well plates. Cells were incubated in full medium for 24 h before the 10^{-6} and 10^{-5} M AST II was added for 48 h. E_2 at the concentration of 10^{-9} M was used as the positive control. After incubated with EdU for 2 h, EdU incorporation was performed on the primary osteoblastic cells. The cells are fixed with 4% paraformaldehyde for 30 min at room temperature. This is followed by Apollo for 30 min at room temperature. Then cells were incubated with Hoechst for 30 min at room temperature followed by PBS wash. Stained sections were examined under a fluorescence microscope (Nikon, Tokyo, Japan). Fluorescence signals were detected using a band-pass filter 555±15 nm for the 550 nm excitation. For each sample, approximately 200 cells were counted and the percentage of EdU-positive cells was calculated.

Measurement of alkaline phosphatase activity. ALP activity was determined by quantitative and staining examination. Osteoblasts cultured in 24-well plates with MEM containing 1% FBS, L-ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) in the presence or absence of agents (10^{-7}-10^{-5} M). E_2 at the concentration of 10^{-9} M was used as the positive control. After 1 to 7 days, cells were gently washed twice with PBS, then lysed with 0.2% Triton X-100 and the lysate was centrifuged at 14,000 x g for 5 min. The supernatant was collected for the measurement of ALP activity by ALP activity assay kit, and protein concentration were determined by BCA-protein assay kit, respectively (33).

Measurement of collagen I and osteocalcin levels. Collagen I and osteocalcin ELISA kits were used to detect collagen I and osteocalcin levels, respectively. Cells were treated with various concentrations (10^{-7}-10^{-5}) of AST II for the indicated times (5-8 days). E_2 at the concentration of 10^{-9} M was used as the positive control. According to the instructions, these samples were placed in 96-well microtiter plates coated with monoclonal detective antibodies and incubated for 2 h at room temperature. After removing unbound material with washing buffer, horseradish peroxidase conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution, with color intensity in proportion to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm. Results are presented as the percentage of change of the activity compared with the untreated control.

Measurement of mineralized matrix. Mineralization in osteoblast cultures was determined by Alizarin Red-S (Aldrich, Milwaukee, WI, USA) staining. Osteoblasts were seeded in 24-well plates at a density of $2x10^5$ cells/well. After 2 days of incubation, cells were washed twice with PBS solution, treated with or without AST II, and cultured with MEM containing 1% FBS, L-ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM), and the medium were changed every 3 days. E_2 at the concentration of 10^{-9} M was used as the positive control. After incubation with and without drugs for 14 days, cells were washed twice with PBS solution at room temperature, fixed with 70% ethanol for 1 h, washed three times with distilled water, then stained with 40 mmol/l Alizarin Red-S (pH 4.2) for 15 min at room temperature. After removing the Alizarin Red-S solution, cultures were washed three times with deionized water and then cultured with PBS for another 15 min. Images of the mineralized matrices were photographed using Nikon microscope. To quantify matrix mineralization, Alizarin Red-S stained cultures were incubated in 100 mmol/l cetylpyridinium chloride (Sigma) for 1 h to solubilize and release calcium-bound Alizarin Red-S into solution. The extracted stain was then transferred to a 96-well plates, and the absorbance of the released Alizarin Red-S was read on ELISA reader at 570 nm (33).

Assaying the levels of secreting BMP-2. Cells were treated with various concentrations (10^{-7}, 10^{-6}, 10^{-5} M) of AST II for 24 h. The culture medium was collected and measured for BMP-2 using BMP-2 ELISA kits, followed the same steps as in section 2.7. Results are presented as the percentage of change of the activity compared with the untreated control.

Analysis for western blotting. To cells treated with or without agents for 24 h, 1 mmol/l PMSF was added and lysed by Nuclear and cytoplasmic protein extraction kit at 4˚C. The protein concentration was determined by BCA-protein assay kit. After obtaining the soluble fraction of nuclear and cytoplasmic protein, respectively, they were used for the western blotting. Equal amounts of protein were subjected to a 15% SDS-polyacrylamide gel electrophoresis. The protein was transferred to nitrocellulose membranes (Pall, NY, USA) using transfer buffer (50 mM Tris, 190 mM glycin, and 10% methanol) at 50 V for 2.5 h. The membranes were incubated with blocking buffer (0.05% Tween-20 and 5% non-fat milk) for 12 h at 4˚C. After washing three times with PBS, the blot was incubated with primary antibody (rabbit polyclonal anti-Smad1/5/8, anti-phosphorylated Smad1/5/8, anti-p38, anti-phosphospecific p38, anti-ERK1/2, anti-phosphospecific ERK1/2, anti-JNK, anti-phosphospecific JNK, anti-Cbfa1, anti-Histone, 1/500 dilution; mouse monoclonal anti-β-actin, 1/1,000 dilution) for 12 h at 4˚C. Subsequently, the membranes were washed three times for 10 min with TBS buffer containing 0.05% Tween-20, and then incubated with anti-rabbit or anti-mouse secondary antibodies (1:5,000) for 1 h at room temperature. The membranes were washed three times for 10 min with TBS buffer, and once for 10 min with PBS. Detection was performed using the enhanced chemiluminescence western blotting detection system (Millipore, MA, USA) (34).

Statistical analysis. Data are expressed as the mean ± SD. Statistical analyses were done by using the one-way ANOVA

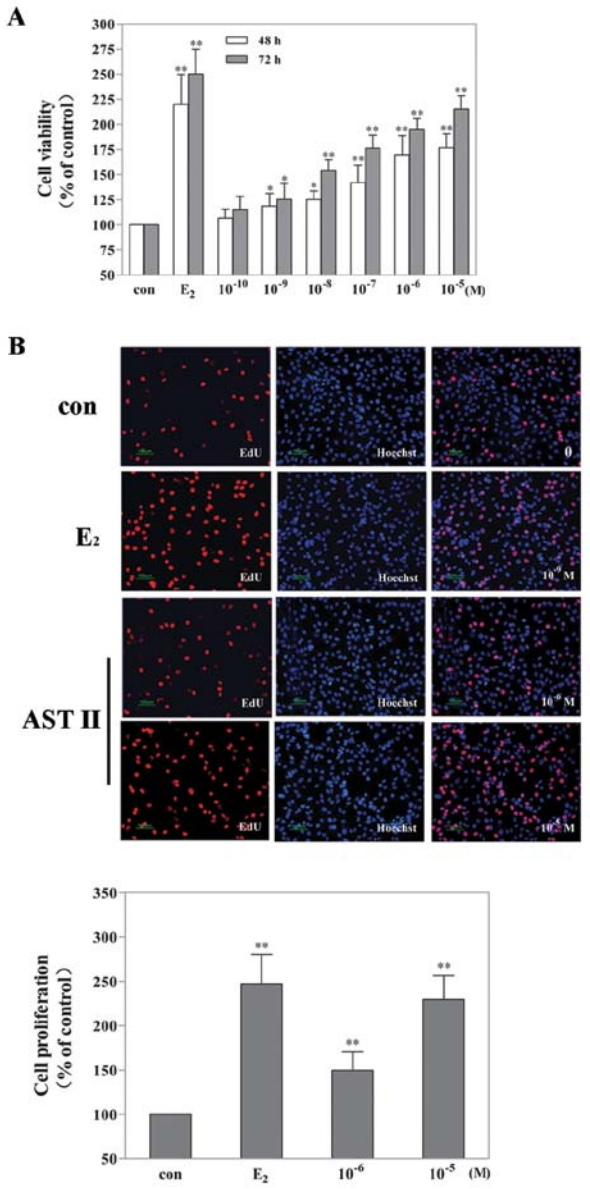

Figure 2. The effects of Astragaloside II (AST II) on the cell viability and proliferation of rat primary osteoblastic cells. Adherent cells that proliferated were incubated with different concentrations (M) of AST II for various time intervals. Cell viability and proliferation were evaluated by (A) the MTT and (B) EdU assay (x200), respectively. For each sample, approximately 200 cells were counted and the percentage of EdU-positive cells was calculated. Estradiol (E_2, 10^{-9} M) was used as the positive control. Values are the mean ± SD of three independent experiments. *P<0.05 as compared with control; **P<0.01 as compared with control (n=5).

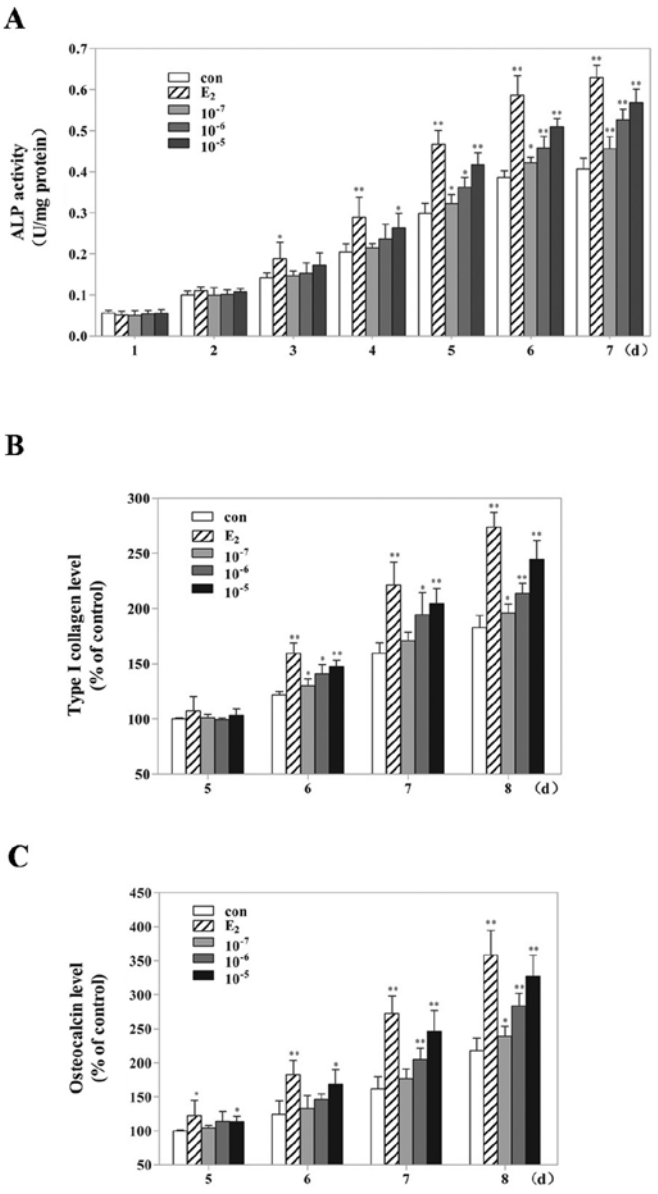

Figure 3. Enhancement of the differentiation of osteoblast by Astragaloside II (AST II). AST II increased (A) ALP activity, (B) type I collagen synthesis, and (C) osteocalcin production. Rat primary osteoblastic cells were treated with various concentrations (M) of AST II for various time intervals. ALP activity was assessed using a commercial ALP kit. The amount of type I collagen and osteocalcin in culture medium were assessed by type I collagen and osteocalcin ELISA kits. E_2 (10^{-9} M) was used as the positive control. *P<0.05 as compared with control; **P<0.01 as compared with control (n=5). The values represent mean ± SD of three independent experiments.

test to compare the different groups. A probability P-value <0.05 was considered to be statistically significant.

Results

AST II promotes the viability of rat primary osteoblasts. We first determined the effect of AST II on the cell viability of rat primary osteoblasts by means of MTT assay. Our results showed that either treatment of osteoblasts with AST II (10^{-10}-10^{-5} M) or E_2 (the positive control) for 48 and 72 h, significantly promoted the cell viability in a concentration-dependent manner (Fig. 2A).

AST II enhances the proliferation of rat primary osteoblasts. The effect of AST II on the cell proliferation of rat primary osteoblasts was evaluated by means of EdU incorporation assay. The percentage of EdU-positive cells, indicating progression into S-phase, was calculated. In the control group, 24% of the cells were EdU-positive, compared with 36% and 55% of the cells treated with 10^{-6} and 10^{-5} M AST II for 48 h, and 59% with 10^{-9} M E_2 (the positive control) (Fig. 2B).

AST II enhances the differentiation of rat primary osteoblasts. ALP activity is a phenotypic marker for the early differentiation of osteoblasts. We assessed the effect of AST II on the

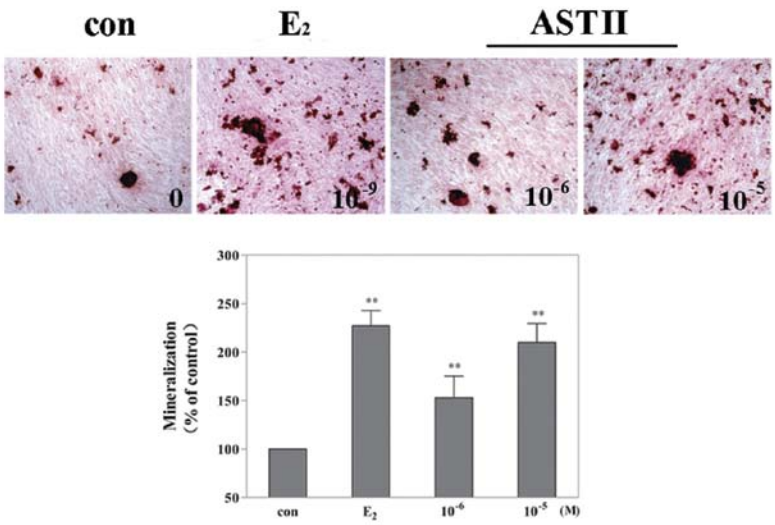

Figure 4. Astragaloside II (AST II) increased the mineralization of osteoblasts. Cells were cultured in 24-well plates and cultured in medium containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM). The cells were treated with or without AST II (10^{-6} M and 10^{-5} M) for 2 weeks. At the end of the experiment, the mineralized nodule formation was assessed by Alizarin Red-S staining (x200). The bound staining was washed with a solution of 100 mmol/l cetylpyridinium chloride and quantified using a Bio-Rad ELISA reader. E_2 (10^{-9} M) was used as the positive control. *P<0.05 as compared with control; **P<0.01 as compared with control (n=5). Values are the mean ± SD of three independent experiments.

activity ALP. The results showed that the first 3 days after treatment of AST II, the ALP activity in osteoblasts was not increased significantly. While from 4-7 days after treatment, the ALP activity was increased by AST II and E_2 (the positive control) respectively, in a concentration-dependent manner (Fig. 3A).

The effect of AST II on the late differentiation of osteoblasts was also assessed by determining the production of osteocalcin and type I collagen proteins. As shown in Fig. 3B and C, the levels of type I collagen and osteocalcin proteins were increased by AST II and E_2 (the positive control) respectively, in a concentration-dependent manner from 5-8 days after treatment.

AST II enhances the maturation of primary osteoblasts. The formation of calcified nodule is one of the markers of the osteoblastic maturation. The calcified nodules appeared bright red by Alizarin Red-S staining (Fig. 4). The results showed that the formation of bone nodules was increased in the osteoblasts treated with AST II at various concentrations for 2 weeks. AST II at 10^{-6} and 10^{-5} M was able to stimulate approximately 153 and 210% increases in the formation of calcified nodules compared to the control. The positive control E_2 at 10^{-9} M could stimulate approximately 227% increase compared to the control.

BMP-2 mediates AST II-induced upregulation of differentiation in primary osteoblasts. It has been demonstrated that BMP-2 plays an important role in the process of bone formation (10). To confirm whether the levels of secreting BMP-2 expression were influenced by the presence of AST II, we examined the expression of the secreting BMP-2 in the presence and absence of AST II using BMP-2 ELISA kits. The results indicated that AST II (10^{-7}, 10^{-6}, 10^{-5} M) significantly increased the secreting BMP-2 protein levels in a concentration-dependent manner after 24 h of treatment (Fig. 5A).

To further examine the role of BMP-2 in AST II-induced cell proliferation and upregulation of differentiation, osteoblasts were pretreated with BMP-2 inhibitor, 100 ng/ml noggin protein for 2 h/day, then 10^{-5} M AST II was added at the indicated time. Noggin directly binds to BMP-2, thereby preventing its interaction with BMP receptor (35). Addition of purified noggin protein did not change cell viability and ALP activity. In contrast, concurrent treatment with noggin did not change cell viability, but significantly diminished the AST II-induced ALP activity (Fig. 6A). Therefore, AST II-enhanced cell differentiation may be operated by a BMP-2-dependent pathway. It seemed that AST II-enhanced cell proliferation was not operated by BMP-2.

BMP-2 is involved in the activation of Smad1/5/8, ERK and p38 in AST II treated primary osteoblasts. Binding of BMP-2 to BMP receptor induces receptor heteromeric complexes and subsequently activates SMADs or MAPKs by phosphorylation (11,12). We assessed the activation of Smad1/5/8, p38, ERK1/2 and JNK MAPKs in AST II treated cells. As shown in Fig. 5B, treatment with AST II did not affect the expression levels of unphosphorylated Smad1/5/8, p38, ERK1/2 and JNK, but did increase the levels of phospho-Smad1/5/8, phospho-p38 and phospho-ERK1/2 after 24 h exposure of osteoblasts to AST II. However, AST II did not significantly affect the level of phospho-JNK.

To determine the role of BMP-2 in AST II induced activation of Smad1/5/8, p38 and ERK1/2 in osteoblasts, osteoblast were pretreated with noggin for 2 h, and then co-treated with 10^{-5} M AST II for 24 h. Results showed that noggin pretreatment abrogated the activation of p38 and Smad1/5/8 induced by AST II (Fig. 6B). However, noggin pretreatment had a lesser effect on ERK1/2 (Fig. 6B). It indicates that BMP-2 signaling is the main path participating in the activation of p38 and Smad1/5/8, but only partly involved in the activation of ERK1/2 induced by AST II.

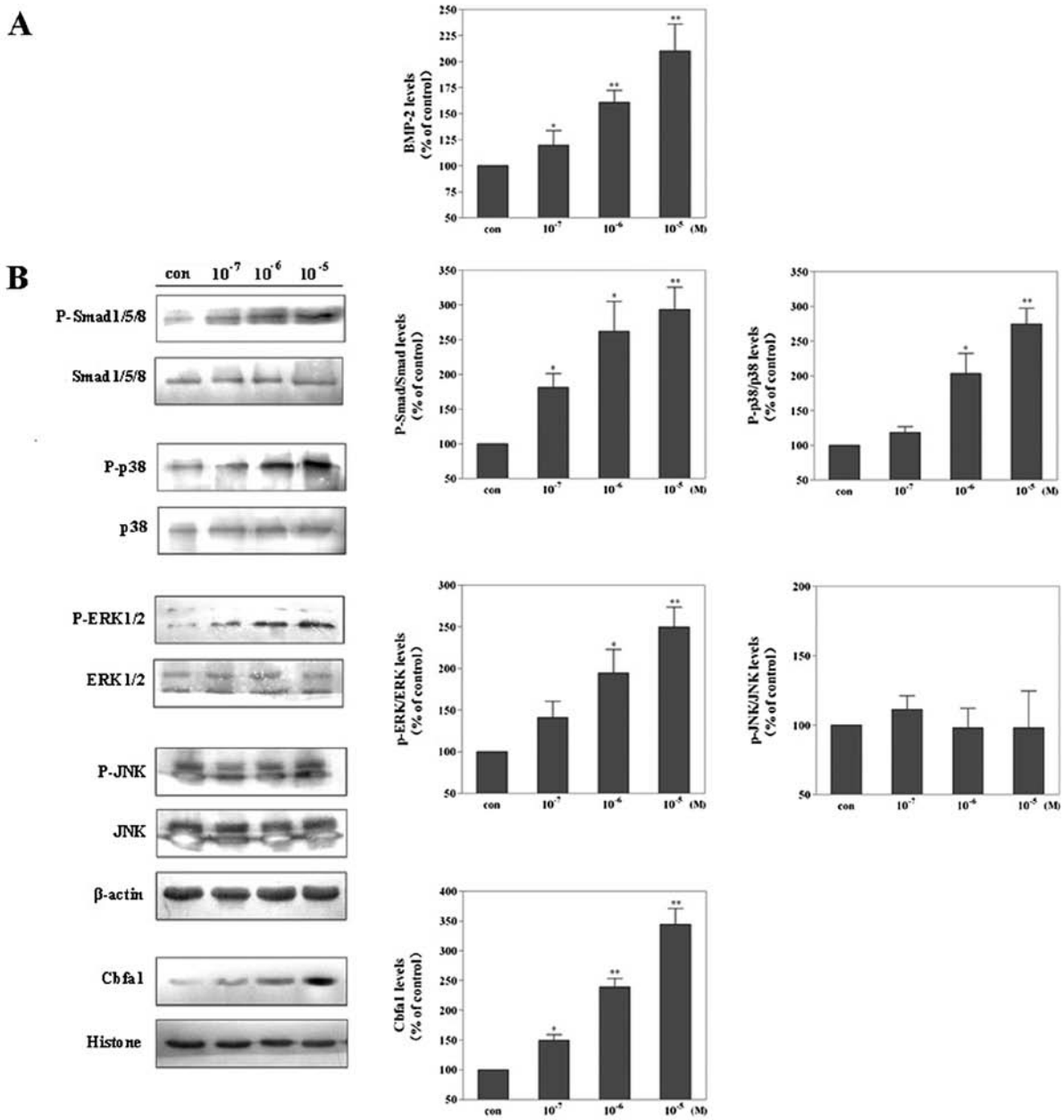

Figure 5. The effects of Astragaloside II (AST II) on the protein levels of (A) BMP-2, (B) Smad1/5/8, p38, ERK1/2, JNK and Cbfa1 in osteoblasts. The protein level of BMP-2 was assessed by BMP-2 ELISA kit. Western blot analysis of Smad1/5/8, p38, ERK1/2, JNK and Cbfa1 expressions after treated with 10^{-7}, 10^{-6} and 10^{-5} M of AST II in osteoblastic cells for 24 h. 'Quantity one' of Bio-Rad was used to analyze the results of western blotting. $^{*}P<0.05$ as compared with control; $^{**}P<0.01$ as compared with control (n=3). The values represent mean ± SD of three independent experiments.

BMP-2 is necessary for the increasing expression of Cbfa1 in AST II treated osteoblasts. Cbfa1 is an essential transcription factor required for osteoblast differentiation (13). After treatment with AST II for 24 h, protein of Cbfa1 in primary osteoblasts was obviously increased (Fig. 5B). To determine the role of BMP-2 in AST II-induced activation of Cbfa1 in osteoblasts, osteoblast were pretreated with or without noggin for 2 h, and then co-treated with 10^{-7} M AST II for 24 h. When osteoblasts were incubated with AST II in the presence of noggin, the stimulatory effect of AST II on Cbfa1 protein was markedly reduced (Fig. 6B). These results suggest that BMP-2 signaling is necessary for the increasing expression of Cbfa1 in AST II-treated primary osteoblasts.

Discussion

The formation of bone involves a complex series of events, including the proliferation and differentiation of osteoblasts and result eventually in the formation of a mineralized extracellular matrix. Since new bone formation is primarily a function of the osteoblast, agents that regulate bone formation act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation and mineralization of the osteoblasts (36). In this research, we examined the effects of AST II on the proliferation, differentiation and mineralization of primary rat osteoblasts. The results show that AST II can stimulate the proliferation, enhance the alkaline phosphatase

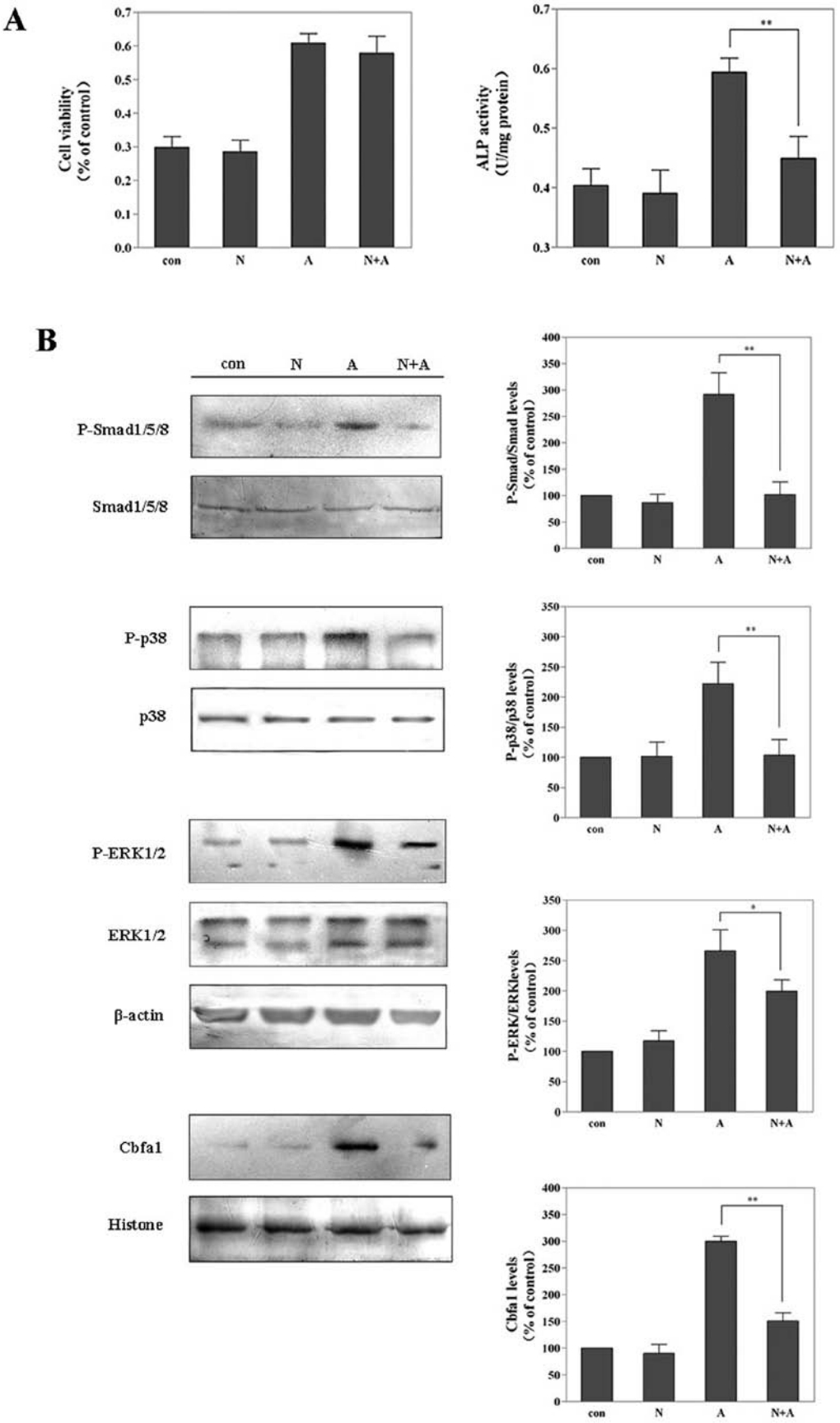

Figure 6. The role of BMP-2 in upregulation of osteoblastic activity by Astragaloside II (AST II) in osteoblasts. (A) Noggin inhibited the induction of AST II on ALP upregulation, but did not affect the induction of AST II on cell viability. (B) Noggin also inhibited the induction of AST II on Smad1/5/8, p38, ERK1/2, Cbfa1 stimulation. (A), Cells were pretreated with or without 100 ng/ml noggin for 2 h/day, and then 10^{-5} M AST II was added. After 3 days, cell viability was evaluated by the MTT assay. ALP activity was assessed using ALP kit after 7 days. (B), Cells were incubated for 2 h in the presence or absence of noggin, and then 10^{-5} M AST II was added and incubated for 24 h. The levels of Smad1/5/8, p38, ERK1/2 and Cbfa1 were determined by western blot analysis. Each value is the mean ± SD of three independent experiments. N, Noggin; A, Astragaloside II.

activity (an early stage marker of differentiation), increase the levels of type I collagen and osteocalcin proteins (late stage markers of differentiation), and promote the formation of bone nodule (a marker of mineralization) in primary rat osteoblasts. Although E_2 as the positive control shows slightly higher effects on the proliferation, differentiation and mineralization of osteoblasts than AST II, the side effects of the long-term use of estrogen, such as a higher incidence of breast carcinoma, endometrial cancer, and cardiovascular disease (21), could not be ignored. Thus, AST II could affect various levels of differentiation process of osteoblast, from early to late stages, which indicated that AST II may be one of the anti-osteoporosis active ingredients of Astragalus.

Then we focused on the signaling pathway of AST II in inducing maturation of osteoblasts. BMPs play an important role in the formation and remodeling of bone by stimulating the differentiation of osteoblast cells (10,31). In the BMP subfamily, BMP-2, which is synthesized and secreted into the extracellular by osteoblasts, has demonstrated a strong osteo-inductive capacity *in vivo* and *in vitro* (6,7). The action of BMPs is mediated by hetero-tetrameric 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 into the nucleus and regulate transcription of target genes associated with differentiation (9). This study indicates that the production of secreting BMP-2 was increased in AST II-treated cells. Also, phosphorylation of Smad1/5/8 is enhanced in AST II-treated osteoblasts.

In addition to Smad activation, BMP-2 can activate Smad-independent pathways, such as MAPK signaling (37,38). BMP-2 can stimulate Ras activity and as a consequence, two MAPKs, ERK and P38 (37,39). P38 and ERK MAPK activation is essential in the BMP-2 upregulation of alkaline phosphatase, type I collagen, osteocalcin, and osteopontin (38). This study observed an increase in p38 and ERK activity in AST II treated cells. These data suggest that activation of p38 and ERK may play an important role on the increase of BMP-2 expression and the cell differentiation by AST II in primary rat osteoblasts. However, the actual mechanism by which AST II operates the two different MAPK signals to regulate different phases of osteoblast differentiation requires further investigation.

The transcription factor, Cbfa1, is a key mediator, required for osteoblastic cell differentiation and ossification (40). It is crucial for regulating expression of bone matrix proteins, such as osteocalcin, type I collagen, osteopontin, and bone sialoprotein (41). It has been reported that both the Smad and MAPK pathways are essential components of the BMP signaling during osteoblast differentiation (11,14-16,42) and for Cbfa1 induction (18). Our study indicates that treating osteoblasts with AST II can promote the expression of Cbfa1 protein, which indicates that AST II may exert its action on bone anabolism through modulating Cbfa1.

What is the role of BMP-2 in the AST II causing a series of changes on osteoblast? As we found in this study, the bone morphogenetic protein antagonist noggin exhibits a suppressive effect against AST II-mediated ALP upregulation, which indicates that the BMP-2 signaling system plays an important role in promoting AST II-mediated cell differentiation. However, it seems that AST II-enhanced cell proliferation is not operated by BMP-2. Furthermore, suppression of BMP-2 signaling by cotreating noggin abrogates Smad1/5/8 and p38 activation in AST II-treated cells, which suggests that the BMP-2 pathway participated in the activation of Smad1/5/8 and p38. However, co-treatment of osteoblast cells with noggin and AST II only partly blocks the activation of ERK1/2. This suggests that the activation of ERK1/2 by AST II may be simultaneously through both BMP-2-dependent and -independent pathways. The increase in Cbfa1 protein by AST II can be prevented by the BMP inhibitor noggin, which demonstrates an involvement of the BMP-2 pathway in the stimulatory effect of AST II on Cbfa1.

Although Radix Astragalus has been used as a traditional Chinese medicine for anti-osteoporosis, it has been used only as healthcare products in the world, because its active ingredients and mechanism of the action are not very clear. The significance for our finding is that AST II as a novel phyto-saponin, promoting osteogenesis activity *in vitro*, which provides scientific evidence and support for the belief in traditional Chinese medicine that *Radix Astragalus* has the ability to strengthen bones and has long been used to treat osteoporosis. This study indicates that *in vitro* AST II is a bone anabolic agent that may exert its osteogenic effects through the induction of BMP-2, Smad-dependent (Smad1/5/8) and -independent (p38 and ERK1/2) signal pathways. All of aforementioned effects may contribute to the induction of osteoblasts proliferation and differentiation.

In conclusion, our study *in vitro* indicates that AST II may become a novel phyto-saponin that is beneficial for stimulating the osteoblastic activity resulting in bone formation. As a phyto-saponin of *Radix Astragalus*, AST II may be a good candidate for promoting bone health, which has not been recognized or reported previously. However, *in vivo* studies are required.

Acknowledgements

This study was supported by the Doctorate Foundation of Northwestern Polytechnical University (grant no. CX201024), the Ministry of Education Fund for Doctoral Students Newcomer Awards of China, and the National Natural Science Foundation of China (grant no. 81073037).

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