Astragaloside-IV modulates NGF-induced osteoblast differentiation via the GSK3β/β-catenin signalling pathway

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Abstract. Astragaloside (AST) is derived from the Chinese herb *Astragalus membranaceus*, and studies have demonstrated that it promotes differentiation of bone marrow-derived mesenchymal stem cells (BMSCs). To the best of our knowledge, however, the functions of the component AST-IV in osteogenesis have not previously been elucidated. The present study aimed to verify the effects of AST-IV in osteogenesis. First, the proliferation and differentiation status of human BMSCs incubated with AST-IV were analysed and compared with a control (no AST-IV treatment). In order to determine the involvement of the glycogen synthase kinase (GSK)3β signalling pathway in AST-IV, overexpression and inhibition of GSK3β was induced during incubation of BMSCs with AST-IV. In order to investigate how neuronal growth factor (NGF) contributes to BMSCs differentiation, BMSCs were co-incubated with an anti-NGF antibody and AST IV, and then levels of osteogenesis markers were assessed. The results demonstrated for the first time that AST-IV contributed to BMSCs differentiation. Furthermore, the GSK3β/β-catenin signalling pathway was revealed to be involved in AST-IV-induced osteogenesis; moreover, AST-IV accelerated differentiation by enhancing the expression levels of NGF. In summary, the present study demonstrated that AST-IV promotes BMSCs differentiation, thus providing a potential target for the treatment of osteoporosis.

Introduction

Osteoporosis is a type of bone disease that decreases bone mass and density, leading to an increased risk of fractures (1,2). It is the most common reason for a broken bone among elderly individuals. Until a break occurs, there are typically no symptoms, and as the disease progresses, the bones may weaken to such a degree that a break may occur spontaneously or as a result of only minor stress. The underlying mechanism of osteoporosis is the imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Osteoclasts are primarily regulated by transcription factor PU.1, and they degrade the bone matrix, whereas osteoblasts participate in rebuilding the bone matrix. Excess degradation results in low bone mass density. Therefore, how to re-establish the balance between osteogenesis and bone degradation in patients with osteoporosis is a clinical focus for researchers.

Numerous cellular signalling pathways are involved in the process of differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) into osteocytes in osteogenesis (3-5). A number of signal transduction proteins, such as NF-κb (6), SMAD1/5/8 (7), bone morphogenetic protein (BMP)/TGF-β (8) and Wnt/β-catenin (9), have been previously reported to participate in osteogenesis.

Previous studies have revealed that enhanced β-catenin activity promotes human osteogenic differentiation, whereas phosphorylation of the Ser 9 site of glycogen synthase kinase (GSK)3β decreases its own activity, which can result in decreased expression levels of β-catenin and increased differentiation of BMSCs (10-12). Moreover, osteogenic differentiation of BMSCs can be potentiated via a GSK3β inhibitor (GI). In addition, evidence has demonstrated that metformin, a drug used to control blood glucose levels, can induce human BMSCs to differentiate into osteoblasts via inhibiting GSK3β and promoting its phosphorylation at Ser9 (13).

To the best of our knowledge, the function of astragaloside (AST) in fracture healing has rarely been studied. Studies have demonstrated that the AST monomer enhances osteogenic differentiation (14-16). Cheng *et al* (14) revealed that AST-I activated the Wnt/β-catenin signalling pathway to stimulate osteogenic differentiation. Kong *et al* (15) demonstrated that AST-II regulated the BMP-1/MAPK and SMAD1/5/8 signalling pathways to induce osteogenic activation of osteoblasts. Li *et al* (17) reported that AST-IV inhibited receptor activator of NF-κB ligand-mediated breakdown of bone tissue by osteoclasts. However, whether AST-IV promotes BMSC osteogenesis has not yet been reported and therefore warrants further investigation. Furthermore, Yin *et al* (18)

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demonstrated that AST exhibited a therapeutic effect on local ischaemia-reperfusion injury and served a neuroprotective role by producing neuronal growth factor (NGF)/TrkA. Previous studies have demonstrated that NGF can phosphorylate Ser9 of GSK3β (19,20). Based on the results of these studies, it was hypothesized that AST-IV may affect the GSK3β/β-catenin signalling pathway via regulating NGF and consequently induce osteogenic differentiation of BMSCs.

The present study investigated the potential functions of AST-IV during osteogenic differentiation of BMSCs, and demonstrated that AST-IV promotes osteoblast differentiation of BMSCs via the GSK3β/β-catenin signalling pathway. Additionally, differentiation of BMSCs induced by AST-IV was revealed to be an NGF-dependent process.

Materials and methods

BMSC culture. Human BMSCs (Cyagen Biosciences, Inc.) were seeded and cultured in T25 culture flasks (Thermo Fisher Scientific, Inc.). Cells were cultured in high glucose DMEM supplemented with 10% foetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine (Sigma-Aldrich; Merck KGaA) and antibiotics [100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.)]. Cells were maintained in an incubator at 37˚C with 5% CO_2.

MTT assay. BMSCs were plated at a concentration of $1x10^4$ cells/ml in 96-well plates. Cells were incubated with 200 μl DMEM per well at 37˚C to allow cells to adhere to the bottoms of the wells. After 24 h, 20 μl MTT solution (5 mg/ml) (Beyotime Institute of Biotechnology) was added to each well. The medium in each well was discarded after 4 h; then, 150 μl DMSO was added, and samples were agitated at a speed of 50 oscillations/min for 10 min at 25˚C. The optical density of each well was measured via a Universal Microplate Reader (Bio-Tek Instruments, Inc.) at 490 nm. Wells containing media without cells were used as internal blank controls.

Alizarin red staining (ARS) and alkaline phosphatase (ALP) activity assay. For ARS, BMSCs were added to 96-well plates at a density of $2x10^5$ cells/well, and following 21 days of AST-IV treatment, cells were cultured and fixed with 70% ethyl alcohol for 1 h at 25˚C. Then, 2% alizarin red (Sigma-Aldrich; Merck KGaA) (pH 8.3) reagent was added to each well for staining at 37˚C for 30 min. Next, 200 μl hexadecylpyridinium chloride solution (0.28 M hexadecylpyridinium chloride, 11.7 mM NaH_2PO_4 in distilled water) was added to wells and incubated for 15 min at room temperature to remove the calcium-bound alizarin red S. Mineralized calcium nodules were observed with a light microscope (magnification, x200).

ALP activity assays were performed following 7 days of AST-IV treatment. BMSCs were washed twice with PBS, and then treated with lysis buffer [1% Triton X-100, 20 mM Tris-HCl (pH, 7.5) and 150 mM NaCl] on ice for 10 min. The ALP activity was detected by ALP Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The activity was detected by measuring the absorbance at 405/650 nm on an absorbance microplate reader (BioTek Instruments, Inc.).

Western blotting. For total protein extraction by RIPA Lysis Buffer (Beyotime Institute of Biotechnology), cells were washed three times with PBS and then incubated for 30 min on ice with cell lysate. Samples were then centrifuged at 4˚C and 11,500 x g for 10 min. The supernatant was subsequently collected and stored in a -80˚C freezer. Then, the protein concentration was measured via a bicinchoninic acid kit. Electrophoresis was performed on 20 μg protein sample on 10% SDS-PAGE. Then, the protein was transferred onto a PVDF membrane. A 5% skimmed non-fat milk solution in tris-buffered saline with 0.1% Tween-20 (TBST) solution was used for blocking the membrane for 1 h at 25˚C. Primary antibodies were incubated with the membrane at 4˚C overnight, and after being washed by TBST three times, the membrane was incubated with secondary antibodies at room temperature for 1 h. The following primary antibodies were used: β-catenin (1:1,000; cat. no. 8480; Cell Signaling Technology, Inc.), Osteocalcin (OCN; 1:1,000; cat. no. ab133612; Abcam), Osteopontin (OPN; 1:1,000; cat. no. ab216406; Abcam); Runt-related transcription factor 2 (Runx2; 1:1,000; cat. no. 8486; Cell Signaling Technology, Inc.), osterix (OSX; 1:1,000; cat. no. ab229258; Abcam), β-actin (1:1,000; cat. no. A5316; Sigma-Aldrich; Merck KGaA), p-S9-GSK3β (1:10,000; cat. no. ab75814; Abcam), GSK3β (1:10,000; cat. no. ab75814; Abcam) and NGF (1:1,000; cat. no. ab68151; Abcam). After probing for 1 h at room temperature with the corresponding secondary antibodies [horseradish peroxidase-conjugated goat anti-rabbit and donkey anti-goat IgG (both 1:5,000; cat. nos. ab205718 and ab97110, respectively; both Abcam)], the protein bands were detected with an ECL kit using a ChemiDoc XRS imaging system. ImageJ software (version 1.52v; National Institutes of Health) was used for densitometric analysis.

Reverse transcription-quantitative (RT-q)PCR. BMSCs were lysed in 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and RNA was extracted using RNeasy Mini kits (Qiagen GmbH). Isolation steps were performed according to the manufacturer's instructions. In order to synthesize cDNA, a PrimeScript™ First Strand cDNA Synthesis kit (Takara Bio, Inc.) was used according to the manufacturer's instructions. Then, qPCR was performed using FastStart Universal Probe Master Mix (Roche Applied Science). The amplification conditions used were as follows: 95˚C for 2 min for denaturation, 30 cycles of denaturation at 95˚C for 30 sec, 55˚C for 30 sec for annealing and 72˚C for 30 sec for extension. Using GAPDH as an endogenous control. The following primers were used: OPN forward, 5'-GATGGCCGAGGTGATAGTGT-3' and reverse, 5'-GTGGGTTTCAGCACTCTGGT-3'; OCN forward, 5'-GGCAGCGAGGTAGTGAAGAG-3' and reverse, 5'-CTAGACCGGGCCGTAGAAG-3'; Runx2 forward, 5'-CGGAATGCCTCTGCTGTTAT-3' and reverse, 5'-TTCCCGAGGTCCATCTACTG-3'; NGF forward, 5'-ATACAGGCGGAACCACACTC-3' and reverse, 5'-AGCCTGGGGTCCACAGTAAT-3'; OSX forward, 5'-GGTCCCCAGTCGAGGAT-3' and reverse, 5'-CTAGAGCCGCCAAATTTGCT-3'; and GAPDH forward, 5'-CCAGGTGGTCTCCTCTGA-3' and reverse, 5'-GCTGTAGCCAAATCGTTGT-3'.

Plasmid transfection and reagents. GSK3β clone plasmid and human anti-NGF antibody were purchased from TsingKe Biotech (Beijing) Co., Ltd. Lipofectamine® 3000

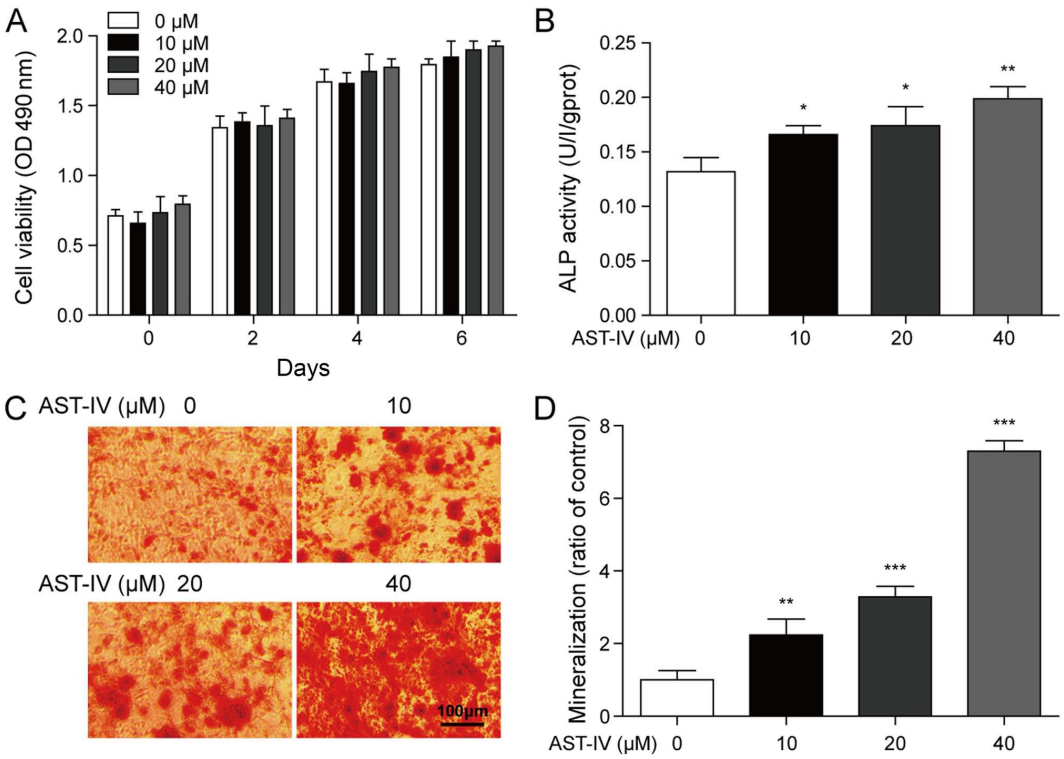

Figure 1. Proliferation and differentiation properties of BMSCs incubated with AST-IV. (A) BMSCs were incubated with gradient concentrations of AST-IV (0, 10, 20 and 40 μM), and cell proliferation was detected at days 0, 2, 4 and 6. (B) Following AST-IV treatment, ALP activity of BMSCs was measured at day 7. (C) Following 21-day treatment with AST-IV (0, 10, 20 and 40 μM), alizarin red S staining was used to detect mineralized nodules in BMSCs (magnification, x200). Scale bar, 100 mm. (D) as a ratio of the Control. n=3; *P<0.05, **P<0.01 and ***P<0.001. BMSCs, bone marrow-derived mesenchymal stem cells; AST-IV, astragaloside-IV; ALP, alkaline phosphatase.

(Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect cells according to the manufacturer's instructions. The ratio of gene/Lipofectamine was 1:3. After 48 h transfection, the cells were collected for subsequent experiments. The GSK3β inhibitor (GI) SB216367 and AST-IV were purchased from Sigma-Aldrich (Merck KGaA) Trading Co. Ltd. Anti-NGF-H0, which does not bind to NGF, was used as a negative control for the anti-NGF antibody. The same dose of DMSO (10 μl) was used as a control for the GI group.

Statistical analysis. Data are expressed as the mean ± standard deviation of three experimental repeats Statistical analysis was performed using unpaired Student's t-test or one-way ANOVA followed by Tukey's post hoc test. Linear association was demonstrated by scatter plots and line of best fit-based linear regression models. P<0.05 was considered to indicate a statistically significant difference.

Results

AST-IV promotes osteogenic differentiation but not proliferation of BMSCs. In order to examine whether AST-IV promotes the proliferation of BMSCs, BMSCs were incubated with BMSC gradient concentrations of AST-IV (10, 20 and 40 μmol/l) and the number of cells was counted. The cell viability of BMSCs are summarized in Fig. 1A, and no significant differences were observed between the AST-IV and control groups. However, ALP activity (day 7) and ARS (day 21) of BMSCs indicated that AST-IV significantly increased BMSC osteogenic differentiation in a dose-dependent manner (Fig. 1B-D). In order to confirm the function of AST-IV in accelerating osteoblastic differentiation of BMSCs, mRNA and protein expression levels of Runx2, OCN, OPN and OSX were determined via RT-qPCR and western blotting following incubation with AST-IV for 7 days (Fig. 2A-F). The results demonstrated that both the mRNA and protein levels of Runx2, OCN, OPN and OSX were significantly increased following treatment, and this increase in expression levels was associated with the concentration of AST-IV.

AST-IV stimulates osteogenic differentiation of BMSCs via GSK3β/β-catenin signalling. AST-IV incubation significantly increased the ratio of p-S9-GSK3β/total GSK3β and protein expression levels of β-catenin in BMSCs (Fig. 3A and B). In order to investigate the function of GSK3β during osteogenic differentiation, a GSK3β-overexpressing vector was transfected into BMSCs. Compared with the empty vector control, the protein expression levels of β-catenin were decreased in AST-IV-treated BMSCs following transfection with the GSK3β overexpression vector (Fig. 3C and D). Overexpression of GSK3β in BMSCs also counteracted the promotion of osteogenic differentiation by AST-IV. ALP activity and mineralization of cells were also decreased in GSK3β-overexpressing BMSCs (Fig. 3E-G). Moreover, GSK3β overexpression decreased the expression levels of Runx2, OCN, OPN and OSX, providing evidence to support the hypothesis that GSK3β signalling affects osteoblastic differentiation of BMSCs (Fig. 3H-J).

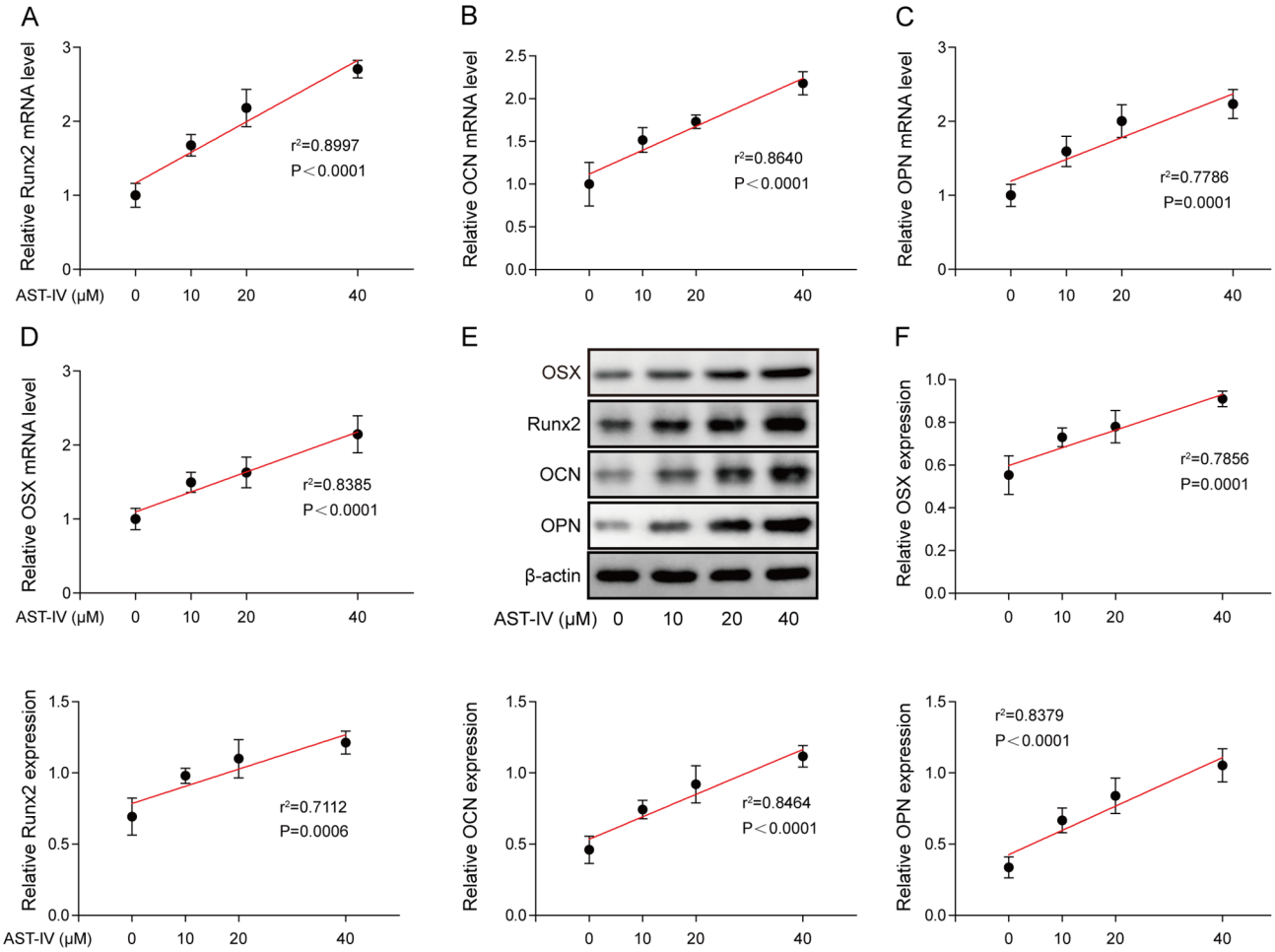

Figure 2. Runx2, OCN, OPN and OSX expression levels in BMSCs following AST-IV incubation. Expression levels of (A) Runx2, (B) OCN, (C) OPN and (D) OSX mRNA in AST-IV-incubated BMSCs (day 7) were detected via quantitative PCR. (E) Runx2, OCN, OPN and OSX protein expression levels were detected by western blotting, with β-actin acting as the endogenous control and (F) linear associations were determined. n=3. BMSCs, bone marrow-derived mesenchymal stem cells; AST-IV, astragaloside-IV.

AST-IV-mediated osteogenic differentiation is dependent on NGF expression levels. Compared with the control group, there was an increase in both mRNA and protein levels of NGF in BMSCs following treatment with different concentrations of AST-IV (Fig. 4A-C). In order to investigate the role of NGF in BMSC osteogenic differentiation, an anti-NGF antibody was added to AST-IV-treated cells and as anticipated it suppressed NGF protein expression levels (Fig. 4D and E). The levels of p-GSK3β and β-catenin were consequently decreased (Fig. 4F and G); the expression levels of Runx2, OCN, OPN and OSX were also decreased in BMSCs following treatment with the anti-NGF antibody (Fig. 4J and L), indicating that NGF directly participated in AST-IV-stimulated BMSC osteogenic differentiation. BMSCs were treated with GI; β-catenin and p-GSK3β levels were increased in the GI group compared with the control group (Fig. 4H and I). Then, cultured BMSCs were co-treated with the anti-NGF antibody, AST-IV and GI. During AST-IV treatment, the mRNA expression levels of Runx2, OCN, OPN and OSX were decreased by treatment with the anti-NGF antibody, while co-treatment with anti-NGF antibody and GI significantly reversed the inhibitory effects of treatment with anti-NGF-alone (Fig. 4J). The protein expression levels of Runx2, OCN, OPN and OSX in the anti-NGF group were decreased compared with those in the anti-NGF-HO group. The protein expression levels of Runx2, OCN, OPN and OSX were increased in the anti-NGF+GI group compared with those of the anti-NGF group (Fig. 4K and L). This indicated that NGF-promoted osteogenic differentiation was dependent on GSK3β/β-catenin signalling.

Discussion

Osteoporosis, or bone mass loss, is one of the most prevalent diseases that affects the elderly population (age, ≥50 years) globally, and it can cause painful fractures (21). Ageing, as well as low body weight, low levels of sex hormones, menopause and smoking, is a risk factor for osteoporosis (22). A decline in osteogenic differentiation capacity of BMSCs is one of the central mechanisms underlying osteoporosis (23). Osteogenic differentiation can replenish bone loss in patients with osteoporosis. However, the cellular and molecular causes underlying decreased osteogenic differentiation capacity of BMSCs in patients with osteoporosis have not yet been fully elucidated.

Certain natural medicines from plants, such as extracts from astragalus, have proven helpful in BMSC osteoblastic

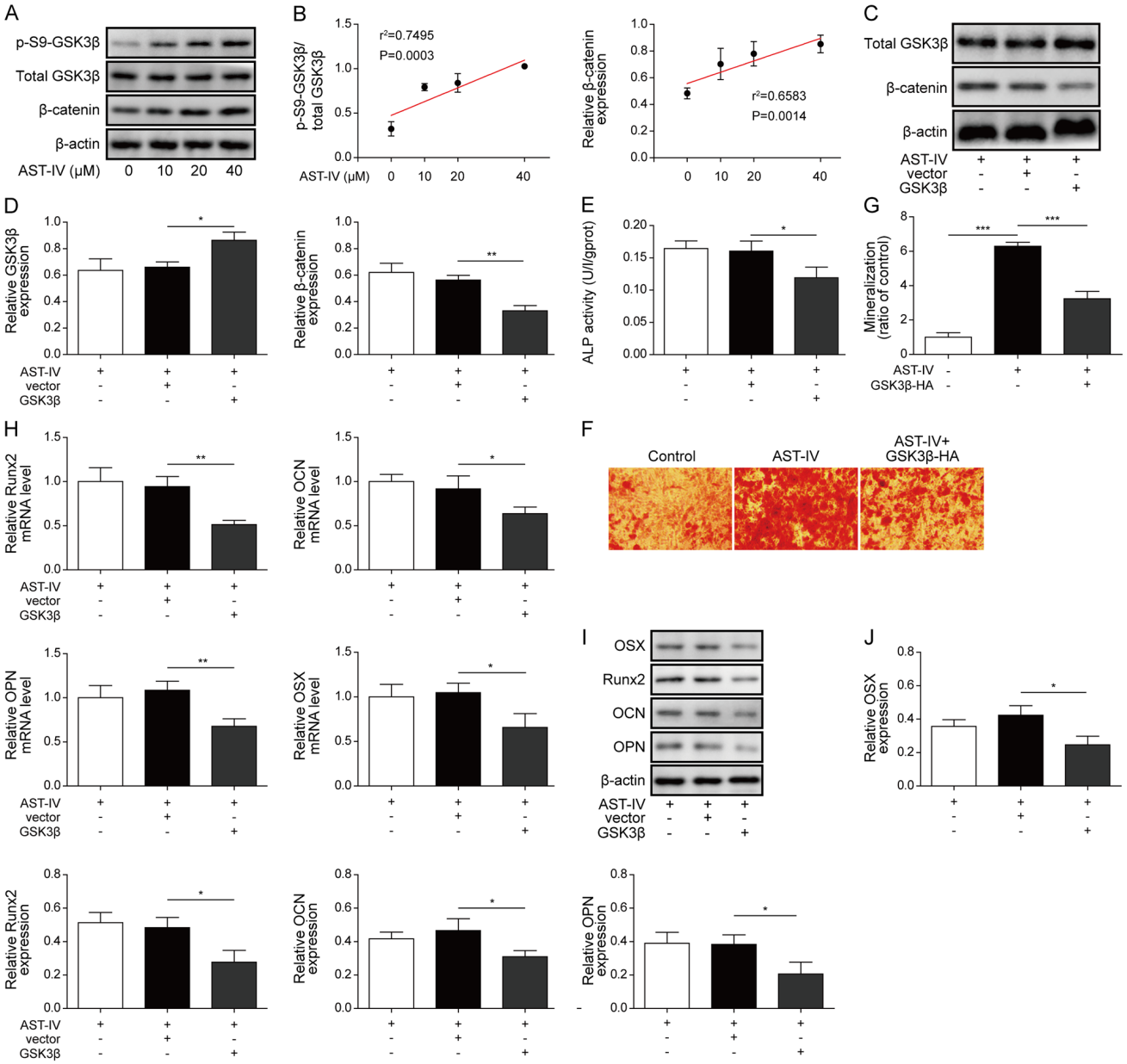

Figure 3. AST-IV stimulates osteogenic differentiation of BMSCs via GSK3β/β-catenin signalling. (A) Western blotting was used to determine the expression levels of p-S9-GSK3β, total GSK3β and β-catenin in untreated BMSCs and BMSCs treated with AST-IV (0, 10, 20 and 40 μM) and (B) linear association was determined. (C) Western blotting was used to detect and (D) quantify the total GSK3β and β-catenin protein changes in BMSCs overexpressing GSK3β or treated with AST-IV (40 μM). β-actin was used as an internal control. (E) ALP activity of GSK3β-overexpressing BMSCs following treatment with AST-IV on day 7. (F) Alizarin red S staining of mineralization conditions in GSK3β-overexpressing BMSCs with AST-IV incubation on day 21 are (G) expressed as a ratio of the Control (magnification, x200). Scale bar, 100 μm. (H) Expression levels of Runx2, OCN, OPN and OSX mRNA following GSK3β overexpression in BMSCs. (I) Runx2, OCN, OPN and OSX protein expression levels were detected via western blotting, with β-actin acting as the endogenous control and (J) quantified. n=3; *P<0.05, **P<0.01 and ***P<0.001. AST-IV, astragaloside-IV; BMSCs, bone marrow-derived mesenchymal stem cells; GSK3β, glycogen synthase kinase 3β; ALP, alkaline phosphatase.

differentiation (14,15). Cheng *et al* reported that AST-I promotes osteoblast differentiation via the Wnt/β-catenin pathway (14). Kong *et al* demonstrated that AST-II induces osteogenic activity of osteoblasts (15). To the best of our knowledge, however, the functions of AST-IV in osteogenesis have not previously been elucidated. Therefore, the present study investigated the role of AST-IV in BMSC osteoblastic differentiation. The experimental results shown that AST-IV increased osteogenesis of BMSCs *in vitro*. The NGF/GSK3β/β-catenin signalling pathway was found to be elevated by AST-IV.

GSK3β/β-catenin has been reported to be a key regulator of osteogenesis (24-27). Pan *et al* (28) demonstrated that conditionally knocking out the transcription factor Yap in mouse osteoblast lineage cells resulted in trabecular bone loss. However, β-catenin overexpression in Yap-deficient BMSCs diminished the osteogenesis deficit. Wang *et al* (29) revealed that adiponectin facilitated BMSC osteogenesis, and that the Wnt/β-catenin pathway was involved in the osteogenic effect of adiponectin. Therefore, the GSK3β/β-catenin signalling pathway is key in promoting osteogenic differentiation. To the best of our knowledge, the present study is the first to

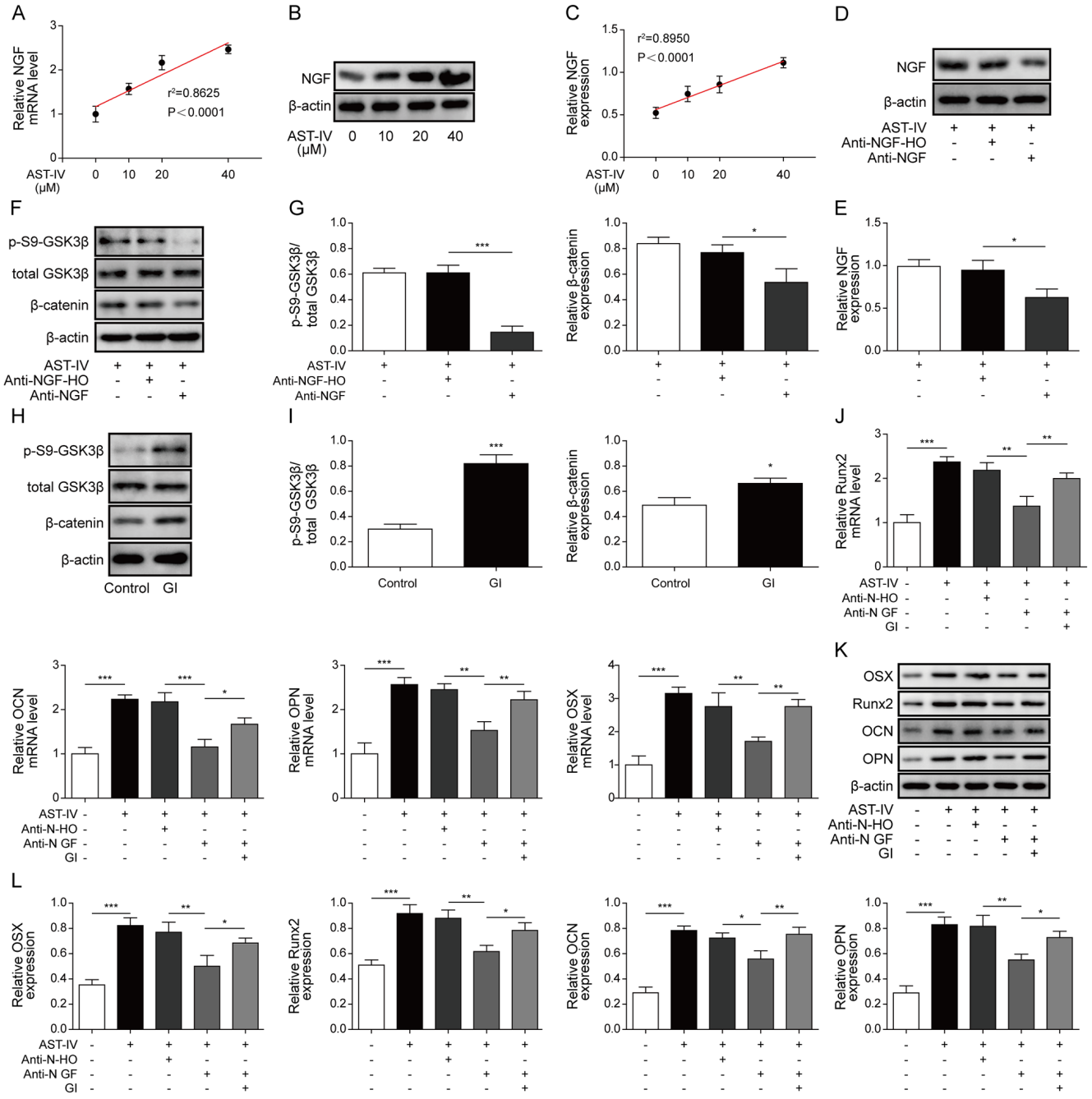

Figure 4. AST-IV-mediated osteogenic differentiation is dependent on NGF expression levels. (A) Expression levels of NGF mRNA in BMSCs following incubation with 0, 10, 20 or 40 μM AST-IV. (B) NGF protein expression levels and (C) linear association between BMSCs incubated with 0, 10, 20 or 40 μM AST-IV. (D) Determination and (E) quantification of NGF protein expression levels in BMSCs following treatment with AST-IV and anti-NGF antibody or anti-NGF-H0 (negative control). (F) Western blotting and (G) relative expression levels of p-S9-GSK3β and total GSK3β in BMSCs following treatment with AST-IV, anti-NGF antibody and anti-NGF-H0. β-catenin was used as a control. (H) Western blotting and (I) relative protein expression levels of p-S9-GSK3β and total GSK3β in GI-treated BMSCs. β-catenin was used as the control. (J) Runx2, OCN, OPN and OSX mRNA levels in AST-IV-cultured BMSCs following GI and anti-NGF treatment. Runx2, OCN, OPN and OSX protein levels were (K) detected and (L) quantified in AST-IV-cultured BMSCs following GI and anti-NGF treatment. n=3; *P<0.05, **P<0.01 and ***P<0.001. AST-IV, astragaloside-IV; NGF, neuronal growth factor; BMSCs, bone marrow-derived mesenchymal stem cells; GSK3β, glycogen synthase kinase 3β; GI, GSK3β inhibitor.

demonstrate that AST-IV induces phosphorylation of GSK3β (Ser-9); this is known to enhance the activity of β-catenin, which promotes BMSC osteogenic differentiation (30,31). In the present study, overexpression of GSK3β resulted in down-regulated β-catenin protein and slowed BMSC differentiation.

NGF is important not only for the development and maintenance of sympathetic and sensory neurons but also for promoting mineralization. This capability makes it a potential target for bone and tissue regeneration. Evidence has indicated that NGF notably contributes to osteogenic differentiation, and it has been assessed both *in vitro* and *in vivo* in different types of cells (32,33). Jin *et al* (30) investigated the effect of biphasic calcium phosphates (BCP) combined with NGF on the growth of osteoblasts *in vitro* and the combined therapeutic effect on the repair of calvarial defects *in vivo*; their results confirmed that the effect of neuro-osteological interactions

via combinatorial treatment with NGF and BCP can promoted osteogenesis and bone formation (30). Another study by Jiang *et al* (31) demonstrated the involvement of NGF signalling in calcification of human articular chondrocytes and the importance of NGF signalling in articular cartilage homeostasis. It has also been reported by Cui *et al* (34) that exogenous treatment with NGF increased ALP levels, as well as ARS and calcium mineral deposition. Yao *et al* (35) reported that local injection of exogenous nerve growth factor increased bone mass, amount of bone trabecula, and bone maturity *in vivo*. Thus, evidence indicates that NGF is involved in osteogenic differentiation of BMSCs. To the best of our knowledge, the present study is the first to demonstrate that AST-IV increases BMSC osteogenesis, which may be a result of upregulated NGF expression levels in BMSCs treated with AST-IV.

The present study demonstrated that both GSK3β/β-catenin signalling and NGF participate in AST-IV-induced osteogenesis and that β-catenin expression levels are negatively affected by GSK3β and positively affected by NGF. A regulatory loop for AST-IV, containing NGF, GSK3β and β-catenin, during BMSC differentiation was identified. AST-IV is upstream of the signalling process that inhibits the function of GSK3β via phosphorylating the Ser9 site; phosphorylated GSK3β induced increased expression levels of β-catenin, resulting in differentiation of BMSCs.

Runx2 and OSX are specific osteoblast transcription factors involved in regulating osteoblast-associated genes. Previous research has demonstrated that OSX may serve a key role in epigenetic regulation of differentiation from MSCs to osteoblasts (36-38). The direct regulatory mechanisms of Runx2 and OSX in regulation of BMSC osteoblastic differentiation by AST-IV require further investigation. In the present study, Runx2 and OSX were increased during AST-IV-induced BMSC osteoblastic differentiation via the NGF/GSK3β/β-catenin axis. The results not only provided an understanding of the molecular pathways involved in regulating NGF-mediated osteogenic differentiation of BMSCs, but also identified potential targets in the treatment of osteoporosis. However, further investigation is required to assess the clinical effectiveness and safety of AST-IV.

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Availability of data and materials

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

Authors' contributions

NYS designed the study. NYS and BD guaranteed the integrity of the study. XLL participated in the conceptualization of the study. XHW, BD and NYS performed the experiments. XHW and JG acquired the data. XHW, NYS and JG conducted the data analysis. JG performed the statistical analysis. NYS prepared and revised the manuscript. XLL and BD reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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