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■ **BONE BIOLOGY**

Angelica polysaccharide promotes proliferation and osteoblast differentiation of mesenchymal stem cells by regulation of long non-coding RNA H19

AN ANIMAL STUDY

X. Xie,
M. Liu,
Q. Meng

Department of Endocrinology, Affiliated Hospital of Jining Medical University, Jining, China.

Objectives

Osteoporosis is a systemic bone metabolic disease, which often occurs among the elderly. Angelica polysaccharide (AP) is the main component of angelica sinensis, and is widely used for treating various diseases. However, the effects of AP on osteoporosis have not been investigated. This study aimed to uncover the functions of AP in mesenchymal stem cell (MSC) proliferation and osteoblast differentiation.

Methods

MSCs were treated with different concentrations of AP, and then cell viability, Cyclin D1 protein level, and the osteogenic markers of runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP-2) were examined by Cell Counting Kit-8 (CCK-8) and western blot assays, respectively. The effect of AP on the main signalling pathways of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and Wnt/β-catenin was determined by western blot. Following this, si-H19#1 and si-H19#2 were transfected into MSCs, and the effects of H19 on cell proliferation and osteoblast differentiation in MSCs were studied. Finally, *in vivo* experimentation explored bone mineral density, bone mineral content, and the ash weight and dry weight of femoral bone.

Results

The results revealed that AP significantly promoted cell viability, upregulated cyclin D1 and increased RUNX2, OCN, ALP, and BMP-2 protein levels in MSCs. Moreover, we found that AP notably activated PI3K/AKT and Wnt/β-catenin signalling pathways in MSCs. Additionally, the relative expression level of H19 was upregulated by AP in a dose-dependent manner. The promoting effects of AP on cell proliferation and osteoblast differentiation were reversed by H19 knockdown. Moreover, *in vivo* experimentation further confirmed the promoting effect of AP on bone formation.

Conclusion

These data indicate that AP could promote MSC proliferation and osteoblast differentiation by regulating H19.

Correspondence should be sent to Q. Meng; email: mengqiang0156@sina.com

Article focus

■ This study aimed to uncover the functions of angelica polysaccharide (AP) in mesenchymal stem cell (MSC) proliferation and osteoblast differentiation.

Key messages

■ AP promotes proliferation and osteoblast differentiation in MSCs.

■ AP activates phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and Wnt/β-catenin signalling pathways in MSCs.

- AP upregulates the expression level of H19 in MSCs.
- AP promotes MSC proliferation and osteoblast differentiation by regulation of H19.
- AP promotes bone formation *in vivo*.

Strengths and limitations

- These results demonstrate the promoting effect of AP on MSC proliferation and osteoblast differentiation by regulation of long non-coding RNA H19 (lncRNA H19). The findings might provide a foundation for further research on the clinical treatment of osteoporosis.
- There is still a lack of clinical trials to confirm the therapeutic effect of AP on osteoporosis. It is still necessary to explore further the extensive application of AP on osteoporosis.

Introduction

Osteoporosis is a systemic bone metabolic disease characterized by low bone mass and the destruction of bone microstructure, which can lead to enhanced bone fragility.[1,2] There are no obvious symptoms of osteoporosis, its major consequence is increasing the risk of bone fracture. The occurrence of osteoporosis is largely due to the different factors that ultimately lead to a decrease in osteoblast activity and to excessive apoptosis.[3,4] Recently, the potential pathogenic genes associated with osteoporosis have been reported, and casein kinase 2-interacting protein-1 (CKIP-1) has been shown to be related to bone formation, and plays a crucial role in the progression of osteoporosis.[5,6] Mesenchymal stem cells (MSCs) have the potential of multidirectional differentiation, which can differentiate into osteoblasts.[7,8] Osteoblasts are important functional cells in the process of bone formation, which is associated with the synthesis, secretion, and mineralization of bone matrix.[9] The balance between proliferation and apoptosis of osteoblasts determines the bone mass.[10] Therefore, the study of the proliferation and differentiation of osteoblasts has great significance for the clinical treatment of osteoporosis.

Angelica sinensis is a commonly used traditional Chinese medicine (TCM). The functions of Angelica sinensis have been widely reported in various diseases, which includes producing blood and anti-tumor, anti-ageing, and anti-inflammation action.[11,12] One study reported the hepatoprotective effect of polysaccharides from different preparations of angelica sinensis.[13] Another interesting study demonstrated the anti-osteoporotic effect of angelica sinensis on ovariectomized rats.[14] Angelica polysaccharide (AP), the main water-soluble component of angelica sinensis, is extracted from the root of angelica sinensis.[15] AP mainly contains D-glucose, D-galactose, D-xylose, L-arabinose, glucuronic acid, and galactic acid.[16] A previous study explored the effects of different methods of extracting AP (tea-making, stewing,

and wine-making), and found that long-boiled soup had the best extraction of AP from angelica.[17] AP possesses wide-ranging pharmacological activities, such as improving the blood system, immune promotion, and anti-tumor action. These activities of AP might exert through affecting asialoglycoprotein receptor (ASGPR)-mediated endocytosis[18] and regulating TLR4.[19] Several studies demonstrated that Morinda officinalis polysaccharide (MOP), Astragalus polysaccharide (APS), and Polygonatum sibiricum polysaccharide (PSP) all have certain preventive and therapeutic effects on osteoporosis.[20-22] However, the effect of AP on osteoporosis has not yet been fully reported.

Long non-coding RNA H19 (lncRNA H19) is one of the most important lncRNAs, and its regulatory functions are widely reported in various cancers.[23,24] One recent study found that lncRNA H19 could suppress adipocyte differentiation of bone marrow MSCs by epigenetic modulation of histone deacetylases.[25] However, it is still unclear whether lncRNA H19 is involved in the regulation of osteoporosis. In the present study, we investigated the effect of AP on MSC proliferation and osteoblast differentiation. The effects of AP on the main signalling pathways of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and Wnt/β-catenin were also explored. Meanwhile, the study uncovered the regulatory effect of lncRNA H19 on AP affecting MSC proliferation and osteoblast differentiation. Furthermore, *in vivo* experiments were performed to uncover the effect of AP on bone formation in an osteoporosis model.[26]

Materials and Methods

Animal and experimental groups. A total of 40 three-month-old female Sprague-Dawley rats were purchased (Shanghai Laboratory Animal Center of Chinese Academy of Sciences, Shanghai, China), and randomly divided into four groups: control; Model; Model+normal saline (NS); and Model+AP. An osteoporosis model was established based on the study by Ezzat-Zadeh et al.[26] Rats in the Model group received an ovariectomy. In the Model+AP group, rats received an ovariectomy, and were treated postoperatively with 400 mg/kg AP for ten days (Sigma-Aldrich, St. Louis, Missouri). In the Model+NS group, rats received an ovariectomy, and were treated postoperatively with 400 mg/kg NS for ten days. Rats in the control group received an ovariectomy but no AP treatment. All rats were raised in cages with water *ad libitum* and the experimental protocols were approved by the ethics committee (No. IACUC-17-041). After completion of modelling for 60 days, bone mineral density (BMD) and bone mineral content (BMC) were measured with a Hologic 2000 Plus densitometer (Hologic, Waltham, Massachusetts). The ash weight and dry weight of femoral bone were measured using an electronic balance.

Cell culture and treatment. The MSCs were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland) and were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, Carlsbad, California), supplemented with 10% foetal bovine serum (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Cells were cultured normally at 37°C in 5% CO_2. The AP (purity ⩾ 95%) was purchased from Sigma-Aldrich. The MSCs were stimulated with different concentrations of AP (100, 200, 300, 400, and 500 μg/ml) for 24 hours. Untreated MSCs served as the control group.

Cell viability assay. The cell viability of MSCs after treatment with AP was determined by a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Gaithersburg, Maryland) assay. Briefly, MSCs (1×10^3 cells/well) were grown in a 96-well plate. After stimulation, the 10 μl CCK-8 solution was added to each well of the 96-well culture plate, and the cultures were incubated for one hour at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The optical density (OD) was measured at 450 nm by using a microplate reader (Bio-Rad Laboratories Inc., Hercules, California, USA).

Cell transfection. The lncRNA H19-specific small interference RNA (siRNA), and its negative control (si-NC), were constructed by GenePharma Co., Ltd. (Shanghai, China) to suppress the relative expression of lncRNA H19. Cell transfections were conducted by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, California) following the manufacturer's protocol. After transfection for 48 hours, cells were harvested for the following study.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs from treated or transfected MSCs were extracted by using Trizol reagent (Life Technologies). The RNA samples were reverse-transcribed by a reverse transcription kit (Takara Biotechnology, Dalian, China). The complementary DNA (cDNA) by the reverse transcription was amplified by using reverse transcription polymerase chain reaction (RT-PCR) with SYBR Green Master Mix (Applied Biosystems, Foster, California). The RT-qPCR assay was performed on Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Daejeon, South Korea). β-actin served as the internal control. Relative quantification analysis was conducted using the $2^{-\Delta\Delta CT}$ method.[27] Each sample was analyzed in triplicate, and all experiments were carried out three times independently.

Western blot assay. The proteins from the treated MSCs were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China), supplemented with protease inhibitors (Roche, Basel, Switzerland). The BCA Protein Assay Kit (Pierce Manufacturing Inc., Appleton, Wisconsin) was used for testing the quantitation of total proteins. Protein samples were separated by utilizing 10% odium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Primary antibodies of cyclin D1 (ab16663), runt-related transcription factor 2 (RUNX2, ab135674), osteocalcin (OCN, ab93876), alkaline phosphatase (ALP, ab83259), bone morphogenetic protein 2 (BMP-2, ab14933), total (t)-PI3K (ab40755), phospho (p)-PI3K (ab182651), t-AKT (ab18785), p-AKT (ab38449), Wnt3a (ab28472), β-catenin (ab16051), and β-actin (ab8227), (all from Abcam, Cambridge, United Kingdom), were incubated with the PVDF membranes at 4°C overnight. The second antibody of horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; ab205718, 1:2000; Abcam) was added and incubated for one hour at room temperature. After this, the Enhanced Chemiluminescent Kit (ECL; Thermo Fisher Scientific, Inc, Waltham, Massachusetts) was used to visualize the protein bands. The intensity of the bands was quantified using Image Lab Software (Bio-Rad Laboratories Inc.).

Statistical analysis. All data from the present study are shown as the mean (SD). Statistical analyses were performed using SPSS 19.0 statistical software (IBM Corp., Armonk, New York). P-values were calculated using a one-way analysis of variance (ANOVA). A p-value < 0.05 was considered to indicate a statistically significant result.

Results

AP promotes MSC proliferation. In this study, MSCs were exposed to different concentrations of AP (0 μg/ml to 500 μg/ml), and MSCs untreated with AP were used as a control group. The cell viability and protein level of cyclin D1 were then assessed by CCK-8 and western blot assays. The results in Figure 1a showed that cell viability was increased by AP at concentrations of 200 μg/ml, 300 μg/ml, and 400 μg/ml ($p < 0.05$), as compared with 0 μg/ml of AP treatment group. Subsequently, 300 μg/ml of AP was used in the next experiments. Western blot analytical results revealed that the protein level of cyclin D1 was notably upregulated by AP in MSCs compared with that in untreated cells ($p < 0.01$; Figs 1b and 1c). The above results suggested that AP could promote cell proliferation in MSCs.

AP promotes osteoblast differentiation. To explore the effect of AP on osteoblast differentiation, we examined four osteogenic markers of RUNX2, OCN, ALP, and BMP-2 in AP-treated MSCs by using western blot. The MSCs untreated with AP were used as a control group. In Figure 2a, the result displayed that the protein level of RUNX2 was obviously increased by AP treatment at day 3, day 7, and day 14 as relative to the control group ($p < 0.05$ or $p < 0.001$). Similarly, the significance increases of OCN, ALP, and BMP-2 after treatment with AP for three, seven, or 14 days ($p < 0.01$ or $p < 0.001$), as presented

Fig. 1a **Fig. 1b** **Fig. 1c**
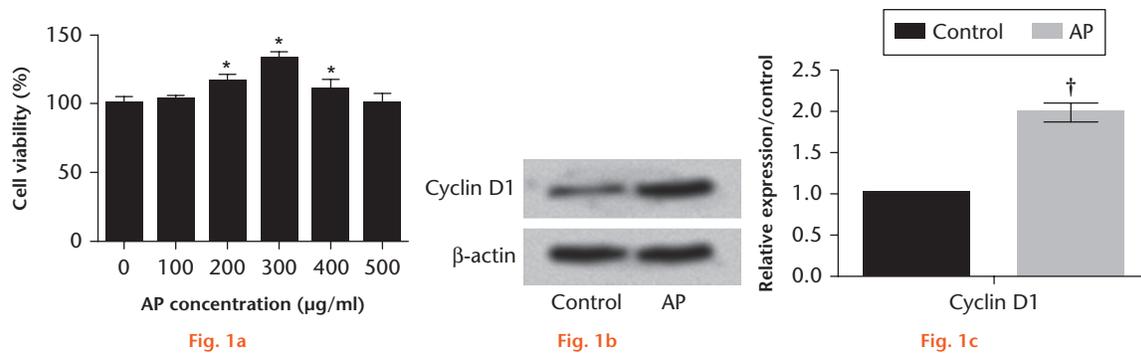
Effect of angelica polysaccharide (AP) on mesenchymal stem cell (MSC) proliferation. a) Different concentrations of AP (0 μg/ml to 500 μg/ml) were used to stimulate MSCs, and untreated cells served as the control group. Cell viability was then detected by Cell Counting Kit-8 (CCK-8) assay. b) and c) MSCs were exposed to 300 μg/ml of AP, and the protein level of cyclin D1 was determined by western blot assay. *$p < 0.05$; †$p < 0.01$.

Fig. 2a **Fig. 2b**

Fig. 2c **Fig. 2d**
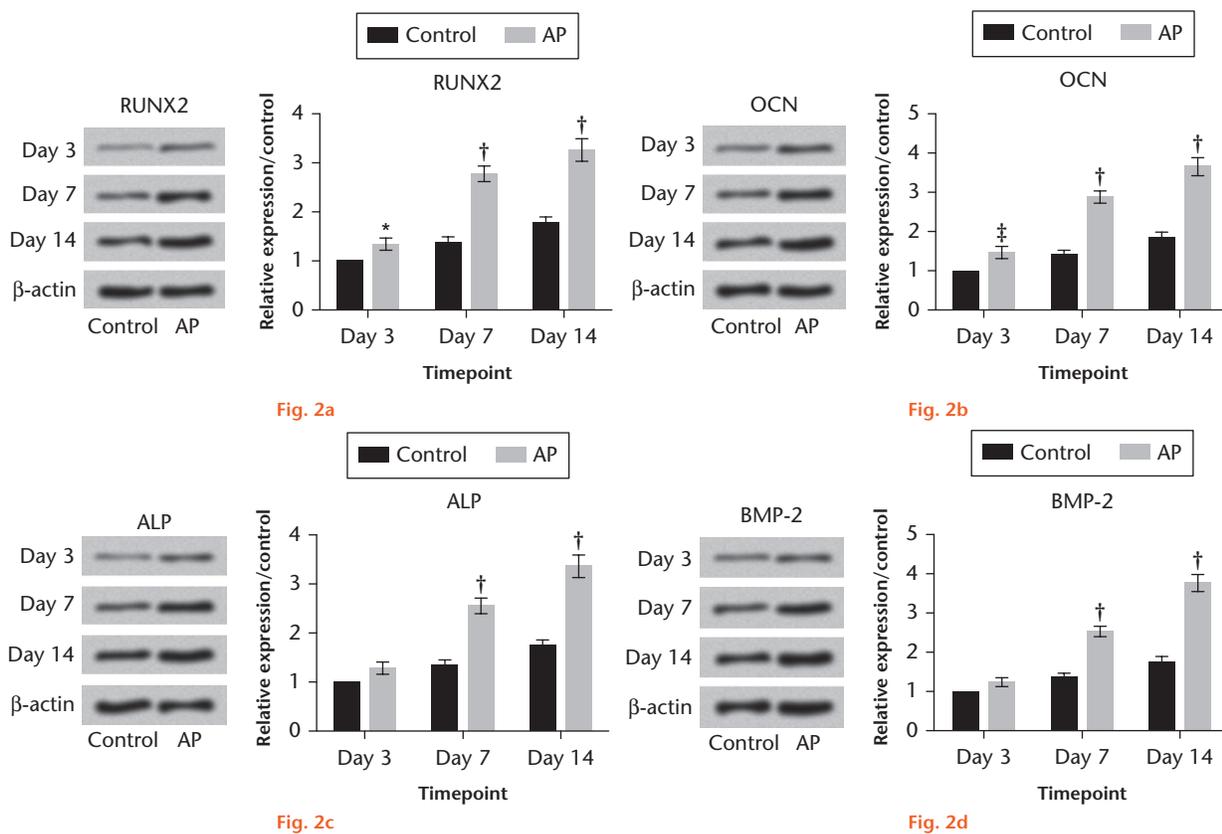
Effect of angelica polysaccharide (AP) on osteoblast differentiation. Mesenchymal stem cells (MSCs) were stimulated with 300 μg/ml of AP, and untreated cells served as the control group. The protein levels of a) runt-related transcription factor 2 (RUNX2), b) osteocalcin (OCN), c) alkaline phosphatase (ALP), and d) bone morphogenetic protein 2 (BMP-2) were measured by western blot at the indicated timepoints of day 3, day 7 and day 14. *$p < 0.05$; †$p < 0.001$; ‡$p < 0.01$.

in Figures 2b to 2d. These data indicate that AP could promote osteoblast differentiation.

AP promotes the activation of PI3K/AKT and Wnt/β-catenin signalling pathways in MSCs. The key signalling pathways of PI3K/AKT and Wnt/β-catenin were then examined after being treated with AP for 24 hours. The MSCs not treated with AP were used as a control group. For the PI3K/AKT signalling pathway, the results in Figures 3a and 3b show that the protein levels of p-PI3K and p-AKT were upregulated by AP in MSCs compared with in the untreated cells

($p < 0.001$). For the Wnt/β-catenin signalling pathway, the results in Figures 3c and 3d showed that the protein levels of Wnt3a and β-catenin were also upregulated by AP in MSCs compared with those in the untreated cells ($p < 0.001$). These data indicate that AP could activate PI3K/AKT and Wnt/β-catenin signalling pathways in MSCs.

AP upregulates the relative expression of H19 in MSCs. The different concentrations of AP (0 μg/ml, 100 μg/ml, 200 μg/ml, and 300 μg/ml) were used to stimulate MSCs, and MSCs untreated with AP were used as a

Fig. 3a

Fig. 3b

Fig. 3c

Fig. 3d
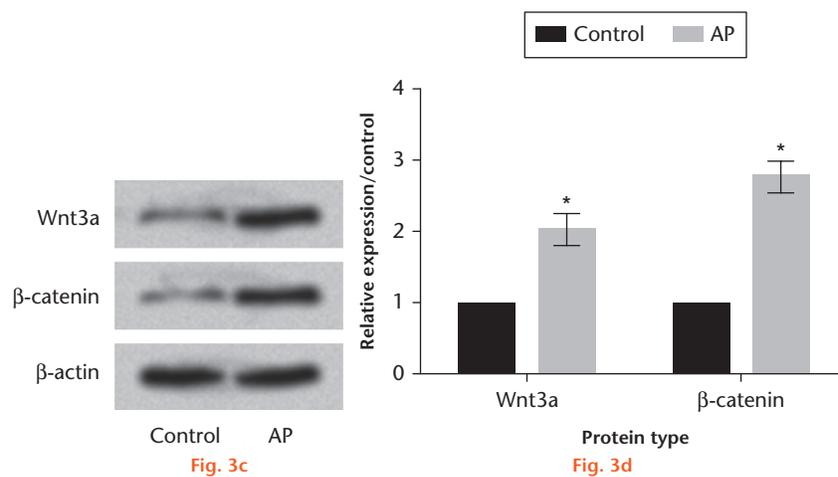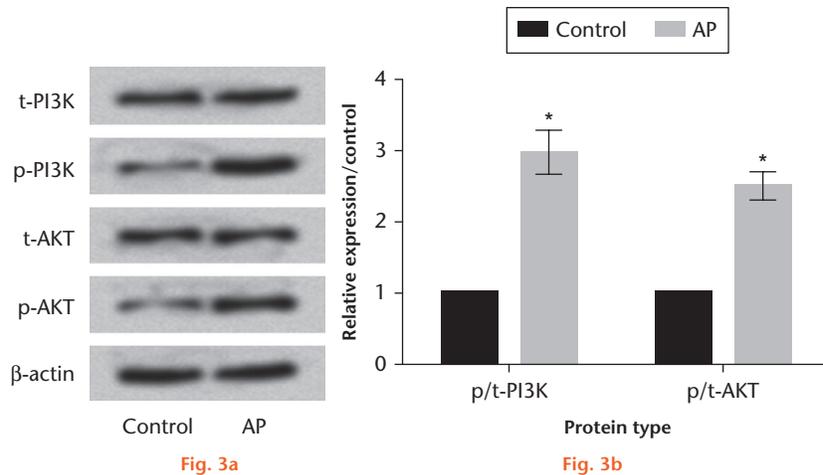
Effect of angelica polysaccharide (AP) on phosphatidylinositol 3-kinase (PI3K)/protein Kinase B (AKT) and Wnt/β-catenin signalling pathways. Mesenchymal stem cells (MSCs) were stimulated with 300 μg/ml of AP, and untreated cells served as the control group. The protein levels of a) and b) phospho (p)/total (t)-PI3K and phospho (p)/total (t)-AKT, and c) and d) Wnt3a and β-catenin were determined by western blot assay. *$p < 0.001$.

Fig. 4
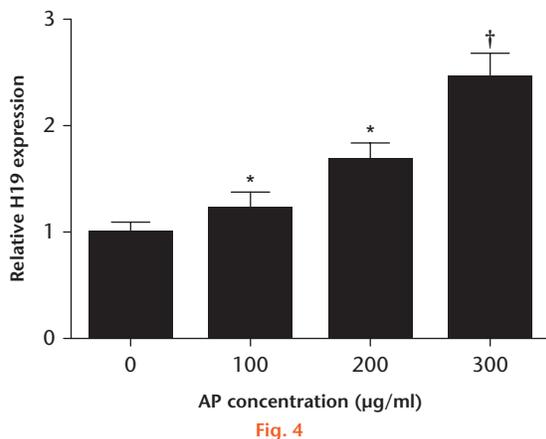
Effect of angelica polysaccharide (AP) on the relative expression of H19. Different concentrations of AP (100 μg/ml to 300 μg/ml) were used to stimulate mesenchymal stem cells, and untreated cells served as the control group. The relative expression level of H19 was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). *$p < 0.05$; †$p < 0.01$.

control group. The relative expression of H19 in these cells was determined by RT-qPCR. The results showed that H19 expression was significantly upregulated by AP at concentrations of 100 μg/ml ($p < 0.05$), 200 μg/ml ($p < 0.05$), and 300 μg/ml compared with the control group ($p < 0.01$; Fig. 4). The results indicate that AP could increase H19 expression level in a dose-dependent manner.

H19 knockdown reverses the promoting effect of AP on cell proliferation in MSCs. To further clarify whether H19 could impact the effect of AP on MSC proliferation, the plasmids of si-H19#1 and si-H19#2 were transfected into MSCs. Cells transfected with si-NC served as a control group. The relative expression of H19 was then determined in these transfected cells. The results in Figure 5a showed that H19 expression level was significantly downregulated in si-H19#1- and si-H19#2-transfecting MSCs compared with that in si-NC-transfecting MSCs ($p < 0.01$), indicating that the plasmids of si-H19#1 and si-H19#2 were successfully transfected in MSCs to suppress H19 expression. The CCK-8 assay results showed that the increase of cell viability induced by AP was inhibited in si-H19#1- or si-H19#2-transfecting MSCs compared with that in si-NC-transfecting MSCs ($p < 0.05$ or $p < 0.01$; Fig. 5b). Additionally, the protein level of
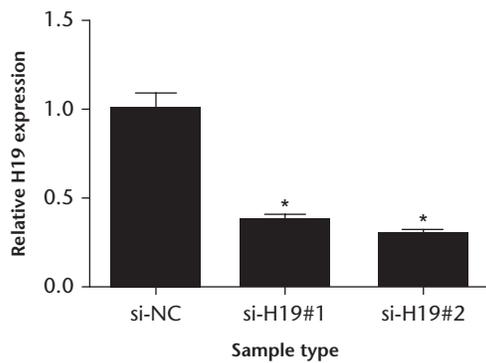
Fig. 5a
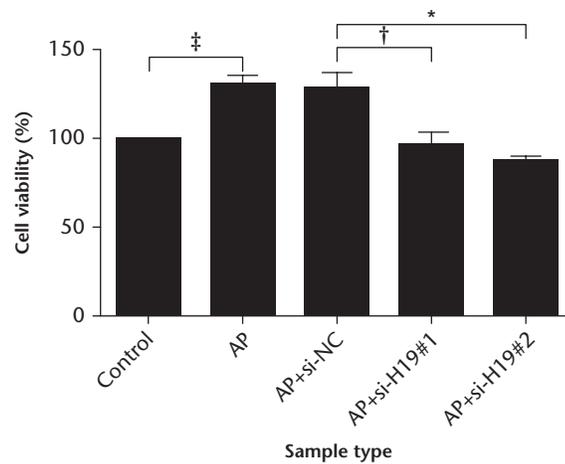
Fig. 5b
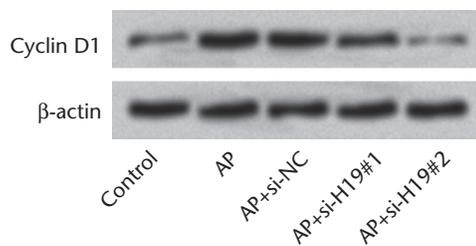
Fig. 5c
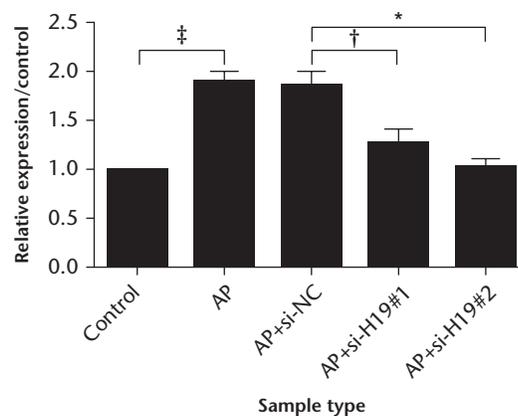
Fig. 5d

Effect of H19 knockdown on cell proliferation in angelica polysaccharide (AP)-treated mesenchymal stem cells (MSCs). a) MSCs were transfected with si-H19#1 and si-H19#2, and MSCs transfected with si-NC served as the control group. The relative expression level of H19 was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR); after transfection and treatment, b) cell viability and c) and d) protein level of cyclin D1 were assessed by Cell Counting Kit-8 (CCK-8) and western blot assays, respectively. *p < 0.01, †p < 0.05: AP+si-H19#1 or #2 *versus* AP+siNC; ‡p < 0.05: AP *versus* control.

cyclin D1 was also inhibited in si-H19#1- and si-H19#2-transfecting MSCs compared with that in si-NC-transfecting MSCs after treatment with AP (p < 0.05 or p < 0.01; Figs 5c and 5d). These results highlight that H19 knockdown could reverse the promoting effect of AP on MSC proliferation.

H19 knockdown reverses the promoting effect of AP on osteoblast differentiation. MSCs were transfected with si-H19#1 and si-H19#2 following treatment with AP. Cells transfected with si-NC followed by treatment with AP served as a control group. The effect of H19 on osteogenic markers of RUNX2, OCN, ALP, and BMP-2 was determined at the timepoints of three days, seven days, and 14 days. The results in Figure 6a showed that the increase of RUNX2 protein level induced by AP was declined markedly by knockdown of H19#1 or H19#2 compared with the si-NC control group (p < 0.001). Similarly, the results in Figures 6b to 6d show that the protein levels of OCN, ALP, and BMP-2 were also inhibited by H19#1 knockdown or H19#2 knockdown in

AP-treated MSCs compared with the si-NC control group (p < 0.05 or p < 0.001). These results indicate that the promoting effect of AP on osteoblast differentiation in MSCs might depend on upregulation of H19.

AP promoted bone formation *in vivo*. A total of 40 rats were randomly divided into four groups: control; Model, Model+NS; and Model+AP. BMD, BMC, and the ash weight and dry weight of femoral bone were observed in these different groups. Results in Figures 7a and 7b reveal that BMD and BMC were both decreased in the Model group compared with the control group (p < 0.05). However, after treatment with AP, we observed that BMD and BMC were significantly increased compared with the Model+NS group (p < 0.05). Additionally, the ash weight and dry weight of femoral bone had similarly decreased in the Model group compared with the control group (p < 0.05). However, in the Model+AP group, the ash weight and dry weight both increased compared with the Model+NS group (p < 0.05; Figs 7c and 7d). These data indicate that AP could promote bone formation *in vivo*.
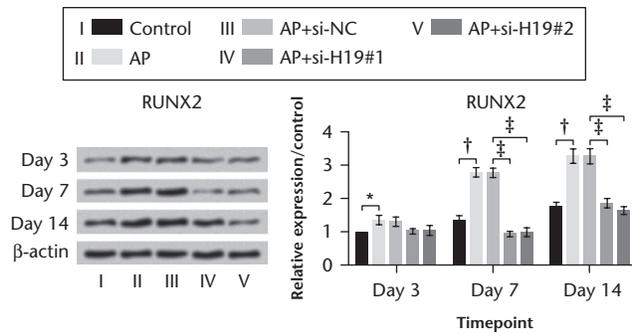
Fig. 6a
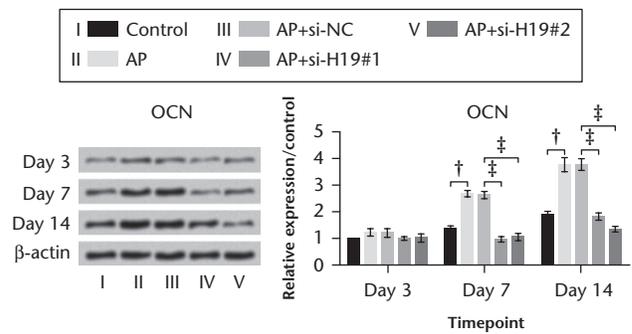
Fig. 6b
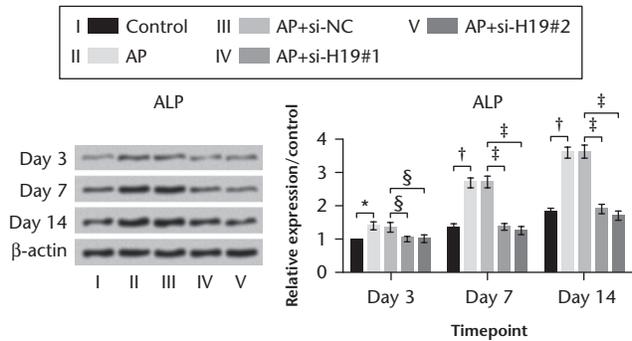
Fig. 6c
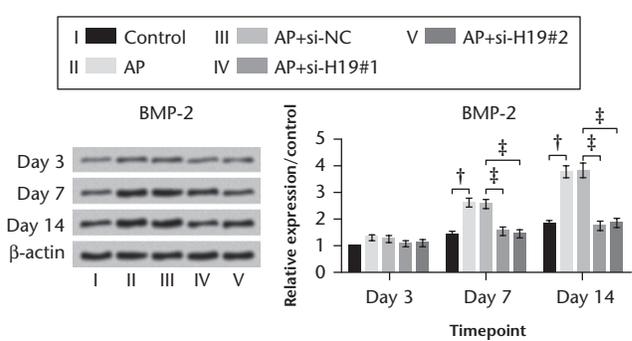
Fig. 6d

Effect of H19 knockdown on osteoblast differentiation in angelica polysaccharide (AP)-treated mesenchymal stem cells (MSCs). The MSCs were transfected with si-H19#1 and si-H19#2, and treated with AP; MSCs transfected with si-NC following AP treatment served as the control group. The protein levels of a) runt-related transcription factor 2 (RUNX2), b) osteocalcin (OCN), c) alkaline phosphatase (ALP), and d) bone morphogenetic protein 2 (BMP-2) were examined by western blot assay at the indicated timepoints of day 3, day 7, and day 14. *$p < 0.05$, †$p < 0.001$: AP *versus* control; ‡$p < 0.001$, §$p < 0.05$: AP+si-H19#1 or #2 vs AP+siNC.

Discussion

As a plant polysaccharide, AP has many advantages such as few side effects and that it can be widely sourced. The extensive pharmacological activities of AP have become key areas of research interest. In the present study, the promoting effects of AP on MSC proliferation and osteoblast differentiation were explored. The results showed that AP promoted cell viability, upregulated cyclin D1, and enhanced RUNX2, OCN, ALP, and BMP-2 protein levels in MSCs. The main signalling pathways of PI3K/AKT and Wnt/β-catenin were also activated by AP. Additionally, we found that the expression level of H19 was upregulated by AP in a dose-dependent manner and the promoting effects of AP on proliferation and osteoblast differentiation of MSCs were reversed by H19 knockdown. Furthermore, *in vivo* experimentation also confirmed the promoting effect of AP on bone formation.

It is well known that many diseases and disorders are associated with osteoporosis.[28,29] Currently, therapeutic treatment for osteoporosis is still limited. Some evidence has shown the positive impact of TCMs on osteoporosis in animal models and in clinical experiments.[30-32] The MSCs are multi-potential stem cells, which can be differentiated into various tissue cells, including osteogenic, chondrogenic, myogenic, and haematopoietic potential stromal cells.[33] MSCs have been widely used to treat various bone diseases.[34] A study from Yan et al[35]

demonstrated that TCM could affect the proliferation of MSCs *in vitro*. RUNX2 is a specific transcription factor in the osteoblastic differentiation of MSCs.[36] Osteocalcin and ALP are extracellular matrix proteins produced by osteoblasts, which are closely associated with the maintenance of bone homeostasis.[37] Bone morphogenetic protein 2 (BMP-2) has been confirmed to initiate osteogenesis.[38] These factors play vital roles in osteoblast differentiation. One study from Su et al[39] found that CYR61 could mediate BMP-2-dependent osteoblast differentiation via $\alpha_v\beta_3$ integrin/integrin-linked kinase/ERK pathway. To the best of our knowledge, the effects of AP on MSC proliferation and differentiation have not been fully investigated. Therefore, based on this theory and research, we explored the effect of AP on MSC proliferation and differentiation. Evidence from Cao et al[40] demonstrated that APS-1d could suppress Henrietta Lacks strain of cancer cells growth in a time- and concentration-dependent manner and exhibited maximal inhibition at a concentration of 300 µg/ml after APS-1d for 72 hours. Based on the study, we explored the effect of AP on cell viability at concentrations of 0 µg/ml to 500 µg/ml. We found that AP promoted cell viability at concentrations of 200 µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml. The maximal promotion of AP on cell viability was presented at a concentration of 300 µg/ml. Thus, 300 µg/ml AP was selected for the following experiments. The subsequent

Fig. 7a

Fig. 7b

Fig. 7c

Fig. 7d
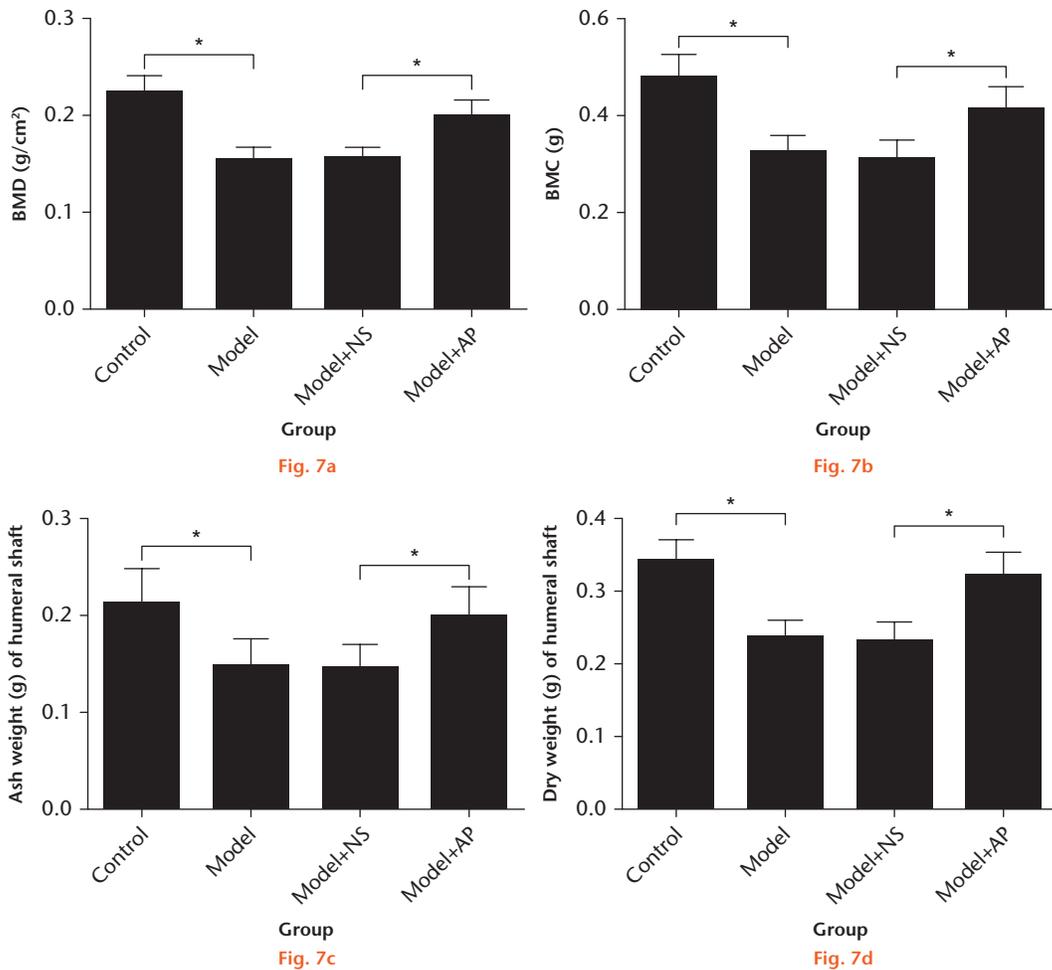
Effect of angelica polysaccharide (AP) on bone formation *in vivo*. A total of 40 rats were randomly divided into four groups: control; Model; Model+NS; and Model+AP (n = 10). a) Bone mineral density (BMD) and b) bone mineral content (BMC) were measured by Hologic 2000 Plus densitometer (Hologic, Waltham, Massachusetts). The c) ash weight and d) dry weight of femoral bone were measured by an electronic balance. *p < 0.05.

experiment results revealed that AP upregulated cyclin D1 expression level, as well as increasing RUNX2, OCN, ALP, and BMP-2 protein levels, indicating the promoting effect of AP on MSC proliferation and differentiation. However, a recent study also showed the inhibitory effects of AP on cell proliferation, migration, and invasion.[41] The results in our study are inconsistent with this study, possibly because of the different cell lines used and the different regulatory mechanisms in the experiments. Further studies are still needed in order to confirm these findings.

Increasing evidence has shown that the PI3K/AKT and Wnt/β-catenin signalling pathways are important regulators in cell proliferation and differentiation.[42,43] One study from Deng and Liu[44] demonstrated that MSCs protected damaged cells by regulation of the PI3K/AKT signalling pathway. Another study showed that Wnt3a could induce the expression of acetylcholinesterase during osteoblast differentiation by regulation of the RUNX2 transcription factor.[45] Additionally, Rossini et al[46] stated that Wnt/β-catenin pathway was involved in the

treatment of osteoporosis. In our study, we explored the effect of AP on PI3K/AKT and Wnt/β-catenin signalling pathways. The results revealed that AP activated PI3K/AKT and Wnt/β-catenin signalling pathways in MSCs. These data indicate that AP may promote proliferation and differentiation of MSCs through activation of the PI3K/AKT and Wnt/β-catenin signalling pathways. Further studies are still needed to clarify this hypothesis.

As an important lncRNA, H19 has been widely reported as participating in the regulation of numerous cellular biological processes.[47,48] It has been confirmed that H19 plays a noticeable role in embryonic placental growth and skeletal muscle differentiation and regeneration.[49,50] One study from Liang et al[51] revealed that H19 could promote osteoblast differentiation and activate Wnt signalling by acting as a competing endogenous RNA, indicating its important role in coordinating osteogenesis. The functions of H19 in MSC differentiation was reported by Huang et al.[52] Additionally, Liao et al[53] reported that H19 could regulate BMP-9-induced osteoblast differentiation of MSCs by mediation of Notch signalling. Based on

these studies, we explored the effect of H19 on AP-promoted MSC proliferation and differentiation. The interesting results revealed that AP increased the expression level of H19 in a dose-dependent manner. However, Yang et al[41] found that expression of H19 was significantly downregulated in AP-treated neuroblastoma cells. Different expression levels of H19 might be due to different cell lines used in the studies. Further research has demonstrated that the promoting effect of AP on MSC proliferation and differentiation is reduced by H19 knockdown. These data indicate that H19 might be an important regulator in the processes of MSC proliferation and differentiation. To confirm further the effect of AP on the pathogenesis of osteoporosis, we constructed an osteoporosis model in ovariectomized rats, and BMD, BMC, and the ash weight and the dry weight of femoral bone were measured. The results showed that AP significantly promoted BMD, BMC, and ash weight and dry weight, indicating that AP might promote bone formation *in vivo*.

Taken together, these results demonstrate the promoting effect of AP on MSC proliferation and osteoblast differentiation by regulation of lncRNA H19 *in vitro*. Additionally, *in vivo* results further demonstrated that AP promoted bone formation in rats with ovariectomy-induced osteoporosis. These findings might provide the research foundation for the treatment of osteoporosis. However, clinical trials that confirm the therapeutic effect of AP on osteoporosis are yet to be seen. Moreover, it is necessary to explore the extensive application of AP in the treatment of osteoporosis.

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Author information
- X. Xie: MM, Associate Chief Physician, Department of Endocrinology, Affiliated Hospital of Jining Medical University; Department of Teaching and Research for Internal Medicine, Clinical Medical College, Jining Medical University, Jining, China.
- M. Liu: MB, Doctor-in-charge, Department of Internal Medicine, Jining Psychiatric Hospital, Jining, China.
- Q. Meng: MM, Doctor-in-charge, Department of Endocrinology, Affiliated Hospital of Jining Medical University, Jining, China.

Author contributions
- X. Xie: Collected and analyzed the data, Performed the experiment, Wrote and revised the manuscript.
- M. Liu: Collected and analyzed the data, Performed the experiment, Wrote and revised the manuscript.
- Q. Meng: Designed the study, Analyzed the data, Revised the manuscript.
- X. Xie and M. Liu are co-first authors on the manuscript.