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BONE BIOLOGY

Astragalus polysaccharide promotes osteogenic differentiation of human bone marrow derived mesenchymal stem cells by facilitating ANKFY1 expression through miR-760 inhibition

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Aims

Astragalus polysaccharide (APS) participates in various processes, such as the enhancement of immunity and inhibition of tumours. APS can affect osteoporosis (OP) by regulating the osteogenic differentiation of human bone mesenchymal stem cells (hBMSCs). This study was designed to elucidate the mechanism of APS in hBMSC proliferation and osteoblast differentiation.

Methods

Reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting were performed to determine the expression of microRNA (miR)-760 and ankyrin repeat and FYVE domain containing 1 (ANKFY1) in OP tissues and hBMSCs. Cell viability was measured using the Cell Counting Kit-8 assay. The expression of cyclin D1 and osteogenic marker genes (osteocalcin (OCN), alkaline phosphatase (ALP), and runt-related transcription factor 2 (RUNX2)) was evaluated using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Mineral deposits were detected through Alizarin Red S staining. In addition, Western blotting was performed to detect the ANKFY1 protein levels following the regulation of miR-760. The relationship between miR-760 and ANKFY1 was determined using a luciferase reporter assay.

Results

The expression of miR-760 was upregulated in OP tissues, whereas ANKFY1 expression was downregulated. APS stimulated the differentiation and proliferation of hBMSCs by: increasing their viability; upregulating the expression levels of cyclin D1, ALP, OCN, and RUNX2; and inducing osteoblast mineralization. Moreover, APS downregulated the expression of miR-760. Overexpression of miR-760 was found to inhibit the promotive effect of APS on hBMSC differentiation and proliferation, while knockdown of miR-760 had the opposite effect. ANKFY1 was found to be the direct target of miR-760. Additionally, ANKFY1 participated in the APS-mediated regulation of miR-760 function in hBMSCs.

Conclusion

APS promotes the osteogenic differentiation and proliferation of hBMSCs. Moreover, APS alleviates the effects of OP by downregulating miR-760 and upregulating ANKFY1 expression.

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Keywords: Astragalus polysaccharide, miR-760, ANKFY1, Osteoporosis, Human bone mesenchymal stem cells

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Article focus

- This study aimed to investigate the molecular mechanisms by which astragalus polysaccharide (APS) affects proliferation and osteogenic differentiation of human bone mesenchymal stem cells (hBMSCs).
- We focused on the influence of APS on the modulation of microRNA (miR)-760

and ankyrin repeat and FYVE domain containing 1 (ANKFY1) expression in hBMSCs.

Key messages

- APS promoted hBMSC proliferation and induced osteogenic differentiation.
- miR-760 was highly expressed in osteoporosis (OP) tissues and neutralized the promotive effect of APS on the osteogenic differentiation and proliferation of hBMSCs.
- APS promotes the proliferation and osteogenic differentiation of hBMSCs by upregulating ANKFY1 expression via the inhibition of miR-760 expression.

Strengths and limitations

- In this study, we proposed for the first time that ANKFY1 expression was downregulated in OP tissues and upregulated by APS in a dose-dependent manner, thereby promoting the osteogenic differentiation and proliferation of hBMSCs in vitro. In addition, through targeting analysis, we elucidated the regulatory role of miR-760 in ANKFY1 expression. The results of rescue analyses suggest that ANKFY1 release may modulate the effects of APS on hBMSC proliferation and osteogenic differentiation induced by miR-760.
- The downstream mechanism of ANKFY1 remains unclear.
- Further in vivo research on APS-mediated regulation of the miR-760/ANKFY1 axis is needed.

Introduction

Osteoporosis (OP) is a metabolic bone disorder. Its incidence increases substantially with the ageing of the population, and results in poor quality of life, increased risk of death, and socioeconomic and medical burdens.[1,2] OP has been reported to be characterized by bone mass reduction and bone tissue microstructure deterioration, and is caused by a decrease in bone formation and an increase in bone resorption.[3] Accumulating evidence indicates that OP is associated with numerous environmental and genetic factors.[4-6] The majority of these factors impact OP development by interfering with the differentiation and activity of osteoblasts.[7,8] Mesenchymal stem cells (MSCs) isolated from adult body tissues have become the preferred source for cell therapy because they are able to differentiate into chondrocytes, adipocytes, or osteoblasts.[9-12] Evidence suggests that MSCs have a reduced ability to differentiate into osteoblasts during the development of OP, resulting in reduced bone formation.[13] Therefore, revealing the molecular mechanism of differentiation of MSCs to osteoblasts is of great importance for the detection of OP and development of novel treatments.

A growing body of research has revealed that polysaccharides extracted from medicinal plants are effective for treating OP.[14,15] Astragalus polysaccharide (APS) is the primary active element in the water extract of Astragalus root and has various pharmacological effects, including anti-inflammatory, antioxidant, and antitumour activity.[16–18] Additionally, evidence suggests that APS is efficacious in OP treatment. For example, APS protects against bone loss by reducing osteocalcin (OCN) and tumour necrosis factor (TNF)-α protein levels in postmenopausal women with OP.[19] In addition, APS greatly inhibits apoptosis and senescence of human bone mesenchymal stem cells (hBMSCs), which are engendered by iron overload induced by ferric ammonium citrate.[20] Therefore, APS may modulate hBMSC proliferation as well as osteogenic differentiation, and may also be involved in OP.

MicroRNAs (miRNAs), containing 20 to 22 bases, are a category of small non-coding RNAs. They affect target gene transcription, thereby affecting cell behaviours such as proliferation, differentiation, and apoptosis.[21] Evidence suggests that investigating the role of miRNAs in OP and osteogenic differentiation of hBMSCs may provide a unique opportunity to develop new diagnostic and therapeutic approaches for OP.[22,23] Recently, it has been reported that miR-760 expression is upregulated in OP tissues and downregulated in osteogenically induced hBMSCs. Silencing of target genes, using miR-760, was found to reduce OP progression in vivo by decreasing bone mineral density (BMD) in OP rats. Furthermore, miR-760 regulates the osteogenic differentiation and proliferation of hBMSCs in vitro.[24] Nonetheless, whether APS can alleviate the effects of OP by modulating miR-760 expression has not been reported.

Ankyrin repeat and FYVE domain containing 1 (ANKFY1), also known as Rabankyrin-5, is a membrane-binding protein possessing a FYVE domain, which can bind to phosphatidylinositol 3-phosphate.[25,26] So far, increasing studies have revealed the function of ANKFY1 in cell biological processes.[26-28] For example, ANKFY1 knockdown was reported to suppress proliferation and migration of human retinal microvascular endothelial cells.[27] ANKFY1 as an adaptor protein of Cullin-3 could regulate angiogenesis of endothelial cells.[29] However, the effects of ANKFY1 on OP or osteogenic differentiation of hBMSCs have not been reported.

This study aimed to investigate the molecular mechanisms by which APS affects hBMSC proliferation and osteogenic differentiation. We focused on the influence of APS on the modulation of miR-760 and ANKFY1 expression levels in hBMSCs. APS therapy may be a promising method for the treatment of OP.

Methods

Clinical samples. Bone tissues were obtained from OP (n = 45) and fracture (n = 45) patients admitted to Wuhan Fourth Hospital (Wuhan, China). All the participants provided written informed consent. The collected bone tissues were stored at -80°C until use. BMD was measured using dual-energy X-ray absorptiometry (QDR 4500W; Hologic, USA). According to the diagnostic criteria published by the World Health Organization, OP is indicated by a BMD T-score greater than -2.5.[30] The

Ethics Committee of Wuhan Fourth Hospital approved this study (approval number: KY 2019-036-01), which was conducted in compliance with the Declaration of Helsinki.[31]

Cell culture and treatment. hBMSCs from SALILA (China) were cultivated in 10% fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) (both Gibco; Thermo Fisher Scientific, USA), and maintained in a 37°C and 5% CO_2 environment. To induce osteogenesis, cells were cultured for 14 days in an osteogenic differentiation medium (Cyagen, China) with α-Minimum Essential Medium (MEM) containing 0.05 mmol/l ascorbic acid, 10 mmol/l β-glycerophosphate, 100 mmol/l dexamethasone (all from Gibco), and 10% FBS.

APS (purity > 95%) was purchased from MilliporeSigma (USA) and incubated for 24 hours with hBMSCs at APS concentrations of 50, 100, 200, and 400 μg/ml (diluted in DMEM). Untreated hBMSCs (not incubated with APS) were used as a negative control (0 μg/ml).

Cell transfection. Small interfering RNAs (siRNAs) targeting ANKFY1 (si-ANKFY1) and scrambled siRNA (si-NC) used in this study were synthesized by GeneChem (China). The miRNA negative controls (mimic-NC and inhibitor-NC), miR-760 mimic, and miR-760 inhibitor were provided by SwitchGear Genomics (USA). hBMSCs were transfected with 75 nM miR-760 mimic, 50 nM miR-760 inhibitor, or 25 nM siRNA for 48 hours with the aid of Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific). The transfection efficiency was assessed using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Cell Counting Kit-8 assay. The Cell Counting Kit (CCK)-8 assay was conducted using the CCK-8 kit (Dojindo, Japan) to evaluate cell viability. APS-treated hBMSCs were cultivated for 24 hours in 96-well plates (1×10^4 cells/well). Thereafter, the cells were supplemented with CCK-8 solution and maintained for one hour at 37°C. A microplate reader (Bio-Rad Laboratories, USA) was used to analyze each well and determine the optical density at 450 nm.

qRT-PCR. A miRNA isolation kit (Ambion, USA) was used for miRNA extraction. The RNeasy Mini Kit (Qiagen, Germany) was used for total RNA extraction. Reverse transcription was performed using the M-MLV Reverse Transcriptase kit (Invitrogen) and TaqMan miRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific). Real-time PCR was performed in a 7300 Real-Time PCR system (Applied Biosystems) using a standard TaqMan MicroRNA Assay (Applied Biosystems) for miRNA and a SYBR Green PCR kit (Toyobo, Japan) for messenger RNA (mRNA). The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantitation of gene expression levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 served as references for the mRNA (ANKFY1, cyclin D1, OCN, alkaline phosphatase (ALP), and runt-related transcription factor 2 (RUNX2)) and miRNA (miR-760), respectively.[32] The primer sequences are listed in Table I.

Alizarin Red S staining. Alizarin Red S (ARS) staining was conducted with cultured hBMSCs (1×10^5 cells) on days 3, 7, and 14 of osteogenic differentiation. At a given point in time, cells were immobilized using 4% paraformaldehyde and stained with 0.1% ARS (MilliporeSigma, USA) for one hour at 37°C. Subsequently, the calcium nodules were observed under a microscope.

Table I. Real-time polymerase chain reaction primer synthesis list.

Gene	Sequences	
miR-760	Forward	5'-CCCCCTCAGTCCACCAGAG-3'
	Reverse	5'-GTTGCATTTCGCTCCCCAC-3'
Cyclin D1	Forward	5'-CCTGGCTGTGGGAACTTCAT-3'
	Reverse	5'-CACCCAAAGCAAGCAGTGTC-3'
ANKFY1	Forward	5'-CACTTCTCGCGAGGTTGCAG-3'
	Reverse	5'-CAGCCACTCTTGTCCACCAT-3'
RUNX2	Forward	5'-TTCAACGATCTGAGATTTGTGGG-3'
	Reverse	5'-GGATGAGGAATGCGCCCTA-3'
ALP	Forward	5'-GAGCGTCATCCCAGTGGAG-3'
	Reverse	5'-TAGCGGTTACTGTAGACACCC-3'
OCN	Forward	5'-GAGGGCAATAAGGTAGTGAA-3'
	Reverse	5'-CATAGATGCGTTTGTAGGC-3'
GAPDH	Forward	5'-AGAAAAACCTGCCAAATATGATGAC-3'
	Reverse	5'-TGGGTGTCGCTGTTGAAGTC-3'

ALP, alkaline phosphatase; ANKFY1, ankyrin repeat and FYVE domain containing 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR, microRNA; OCN, osteocalcin; RUNX2, runt-related transcription factor 2.

Luciferase assay. To construct the wild-type (WT) reporter plasmid, pGL3 plasmids (GeneChem) were inserted with WT ANKFY1 with a putative miR-760-binding sequence. A GeneTailor Site-Directed Mutagenesis System kit (Invitrogen) was used to mutate the ANKFY17 3'-untranslated region (UTR) containing an miR-760-binding site to serve as MUT1, MUT2, and Co-MUT, based on the mutation of the different sites. Lipofectamine 3000 was used to transfect each reporter plasmid with WT, MUT1, MUT2, and Co-MUT or empty vector along with either an miR-760 mimic or mimic-NC. After 48 hours, the Luciferase Reporter System (Promega, USA) was used to detect the luciferase activity in each reporter plasmid.

Western blotting. Total protein from the cells was extracted using RIPA buffer (Roche, Germany). The protein levels were then evaluated using a BCA Kit (Beyotime, China). Subsequently, the proteins were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). Tris-HCl-Tween (TBST) containing 5% BSA was used to block the membranes. The membranes were then incubated overnight at 4°C with GAPDH (ab8245, 1:2,000, Abcam) and ANKFY1 (ab169273, 1:1,000, Abcam, USA) antibodies. On the following day, a secondary antibody (ab97051, 1:1,000, Abcam) was added to the membranes, followed by one hour of incubation. Immune complexes were detected using enhanced chemiluminescence (ECL) (Millipore), and a MyECL Imager (Thermo Fisher Scientific) was used to visualize and quantify the blots.

Statistical analysis. Data from at least three separate experiments, indicated as mean (standard deviation (SD)), were analyzed using GraphPad Prism software (version 6.0; GraphPad Software, USA). One-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference post hoc test, were employed for multigroup analysis, while the independent-samples *t*-test was used for two-group analysis. The Pearson correlation coefficient was used to evaluate the correlation between two variables. Statistical significance was set at $p < 0.05$.

Results

APS promoted hBMSC proliferation and induced osteogenic differentiation. To determine the effect of APS on hBMSC viability, hBMSCs were exposed to different concentrations (0 to 400 μg/ml) of APS. CCK-8 assay results revealed that the viability of the hBMSCs improved with the increase in APS concentration at 100, 200, and 400 μg/ml. However, relative to their viability at 200 μg/ml, hBMSCs showed a decrease in viability at 400 μg/ml APS (Figure 1a). Therefore, the optimal incubation concentration of APS was determined to be 200 μg/ml. Cyclin D1 was used to assess hBMSC proliferation. The results showed that cyclin D1 expression levels in the APS-treated hBMSC group were upregulated 1.93-fold (Figure 1b). Next, the expression levels of osteogenic marker genes (OCN, ALP, and RUNX2) were measured to study the effect of APS on the osteogenic differentiation

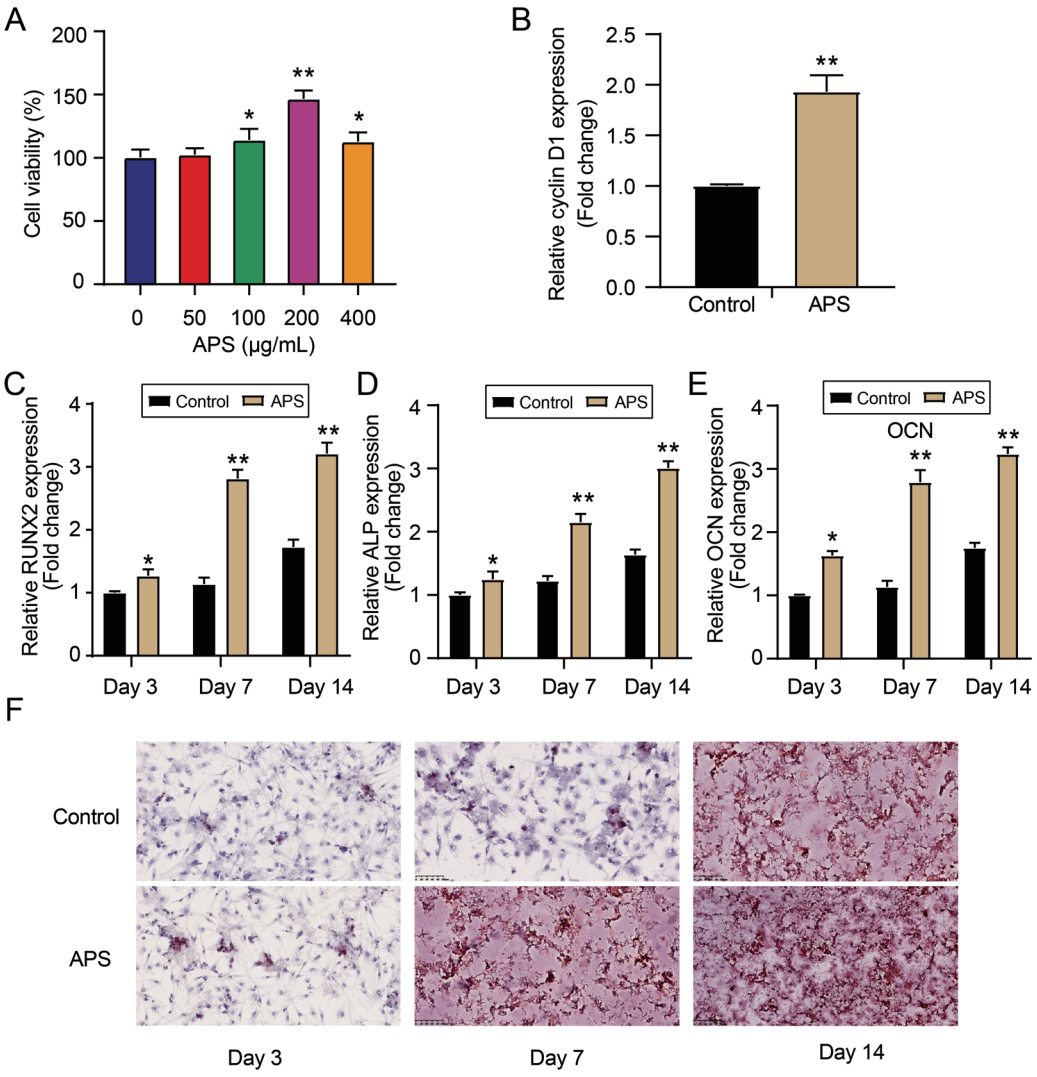

Fig. 1

Astragalus polysaccharide (APS) promoted human bone mesenchymal stem cell (hBMSC) proliferation and induced osteogenic differentiation. a) hBMSCs were stimulated with APS (0, 50, 100, 200, and 400 μg/ml). Cell viability was then detected via Cell Counting Kit-8 assay. *$p < 0.05$ and **$p < 0.001$ versus 0 μg/ml. b) hBMSCs were treated with 200 μg/ml of APS, and the cyclin D1 messenger RNA (mRNA) levels were assessed via quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). **$p < 0.001$ versus control. c) to e) hBMSCs were treated with 200 μg/ml of APS. c) Runt-related transcription factor 2 (RUNX2), d) alkaline phosphatase (ALP), and e) osteocalcin (OCN) mRNA levels were measured via qRT-PCR at days 3, 7, and 14 of osteogenic differentiation. *$p < 0.05$ and **$p < 0.001$ versus control. f) hBMSCs were stimulated with 200 μg/ml of APS, and the calcium nodules were detected by means of Millers Elastic Stain at days 3, 7, and 14 of osteogenic differentiation of hBMSCs. Magnification: 400×. All p-values were calculated using one-way analysis of variance, followed by Tukey's Honest Significant Difference post hoc test.

of hBMSCs. The qRT-PCR results revealed that RUNX2, ALP, and OCN mRNA levels were upregulated by factors of 1.27, 1.25, and 1.63, respectively, in hBMSCs treated with 200 μg/ml APS on day 3; 2.47, 1.78, and 2.46, respectively, in hBMSCs treated with 200 μg/ml APS on day 7; and 1.86, 1.84, and 1.85, respectively, in hBMSCs treated with 200 μg/ml APS on day 14 of osteogenic differentiation (Figures 1c to 1e). Furthermore, ARS staining revealed an increase in the number of calcium nodules among the hBMSCs at days 3, 7, and 14 of osteogenic differentiation (Figure 1f). These data suggest that APS promotes the osteogenic differentiation and proliferation of hBMSCs.

miR-760 was overexpressed in OP tissues and abated the promotive effect of APS on the osteogenic differentiation and proliferation of hBMSCs. The expression levels of miR-760 in the bone tissue of OP and fracture patients without OP were investigated. qRT-PCR revealed upregulation of miR-760 expression by nearly 1.65-fold in the bone tissues of OP patients (Figure 2a). Furthermore, miR-760 expression levels in hBMSCs were decreased by 23% and 48% when the APS concentration was increased to 100 and 200 μg/ml, respectively (Figure 2b). These results indicate that miR-760 may be related to OP and may mediate osteogenic differentiation in hBMSCs when regulated by APS. To further understand whether miR-760 and APS affect hBMSC differentiation and proliferation, the miR-760 inhibitor and mimic plasmids were transfected into hBMSCs. The results demonstrated that the expression of miR-760 was upregulated 2.74-fold in the mimic group and downregulated by 59% in the inhibitor group, indicating that miR-760 expression in hBMSCs was successfully modulated (Figure 2c). The CCK-8 assay results showed that the viability of hBMSCs treated with APS + mimic and APS + inhibitor was 70% and 150%, respectively, compared to the viability of hBMSCs treated with APS alone (Figure 2d). Additionally, qRT-PCR analysis revealed that cyclin D1 expression was downregulated by 33% in the APS + mimic group. Meanwhile, cyclin D1 expression in the APS + inhibitor group was upregulated 1.27-fold (Figure 2e). Furthermore, the detection of key genes for osteogenic differentiation revealed the downregulation of RUNX2, ALP, and OCN expression at different times during the osteogenic differentiation of the APS + mimic group compared with the APS + mimic NC group. Meanwhile, miR-760 interference induced a higher level of osteogenic differentiation than that in the APS + inhibitor NC group (Figures 2f to 2h). These results suggest that knockdown of miR-760 enhances the promotive effect of APS on osteogenic differentiation and proliferation of hBMSCs. Moreover, the upregulation of miR-760 expression counteracted the effects of APS.

miR-760 directly targeted ANKFY1. We analyzed the downstream genes associated with miR-760 expression. The sequence relationship between miR-760 and ANKFY1 was predicted using TargetScan (Figure 3a).[31,33] The luciferase activity assay results showed that hBMSCs carrying the miR-760 mimic along with the WT, MUT1, or MUT2 sequence had an approximately 60%, 40%, and 25% decrease in luciferase activity, respectively. Meanwhile, hBMSCs with miR-760 overexpression and Co-MUT showed no changes in luciferase activity, suggesting that ANKFY1 may be directly targeted by miR-760 (Figure 3b). Analysis of ANKFY1 expression levels in clinical samples showed that its levels in OP tissues were downregulated by 58% (Figure 3c). Moreover, the expression levels of ANKFY1 in OP tissues were negatively correlated with miR-760 expression levels (Figure 3d). These results suggest that ANKFY1 may participate in OP as a downstream target of miR-760. Therefore, we explored the effect of APS on ANKFY1 expression levels. Figure 3e shows that ANKFY1 expression levels in hBMSCs treated with APS increased 1.44-fold at 100 μg/ml and 1.82-fold at 200 μg/ml. Additionally, we observed a 3.02-fold upregulation of ANKFY1 mRNA expression following the knockdown of miR-760 and 62% downregulation after miR-760 overexpression (Figure 3f). Similarly, relative to the corresponding groups, Western blotting showed that ANKFY1 protein levels were upregulated by approximately 150% in the inhibitor group and downregulated by approximately 50% in the mimic group (Figure 3g). These results suggest that miR-760 negatively regulates ANKFY1 expression.

APS promotes the proliferation and osteogenic differentiation of hBMSCs by facilitating ANKFY1 expression via miR-760 inhibition. It is unclear whether ANKFY1 influences the effects of miR-760 on hBMSCs. Hence, we transfected hBMSCs with si-ANKFY1 and miR-760 inhibitors to investigate the changes in cell viability and differentiation-related factors. An approximate 2.89-fold increase and 71% decrease in ANKFY1 expression levels were detected after transfection with the miR-760 inhibitor and si-ANKFY1, respectively (Figure 4a). Additional transfection with si-ANKFY1 completely offset the promotive effect of the miR-760 inhibitor on ANKFY1 expression levels (Figure 4a). In addition, our results indicated that low ANKFY1 expression levels diminished cell viability, inhibited cyclin D1 expression by 27%, and partially eliminated the promotive effect of miR-760 knockdown on cell survival (Figures 4b and 4c). The introduction of si-ANKFY1 decreased the expression levels of RUNX2, ALP, and OCN on days 7 and 14 of osteogenic differentiation in the APS-treated hBMSCs (Figure 4). Moreover, si-ANKFY1 neutralized the miR-760-inhibitor-induced osteogenic differentiation of hBMSCs (Figures 4d to 4f). Our data indicate that through its interaction with miR-760, ANKFY1 participates in the APS-mediated regulation of the functional changes induced by miR-760 in hBMSCs.

Discussion

As a plant polysaccharide, APS has been extensively studied because of its low toxicity and lack of residue.[34] In this study, we focused on the impact of APS on the osteogenic differentiation and proliferation of hBMSCs. The results showed that APS enhanced the viability of hBMSCs, upregulated cyclin D1 expression, boosted the

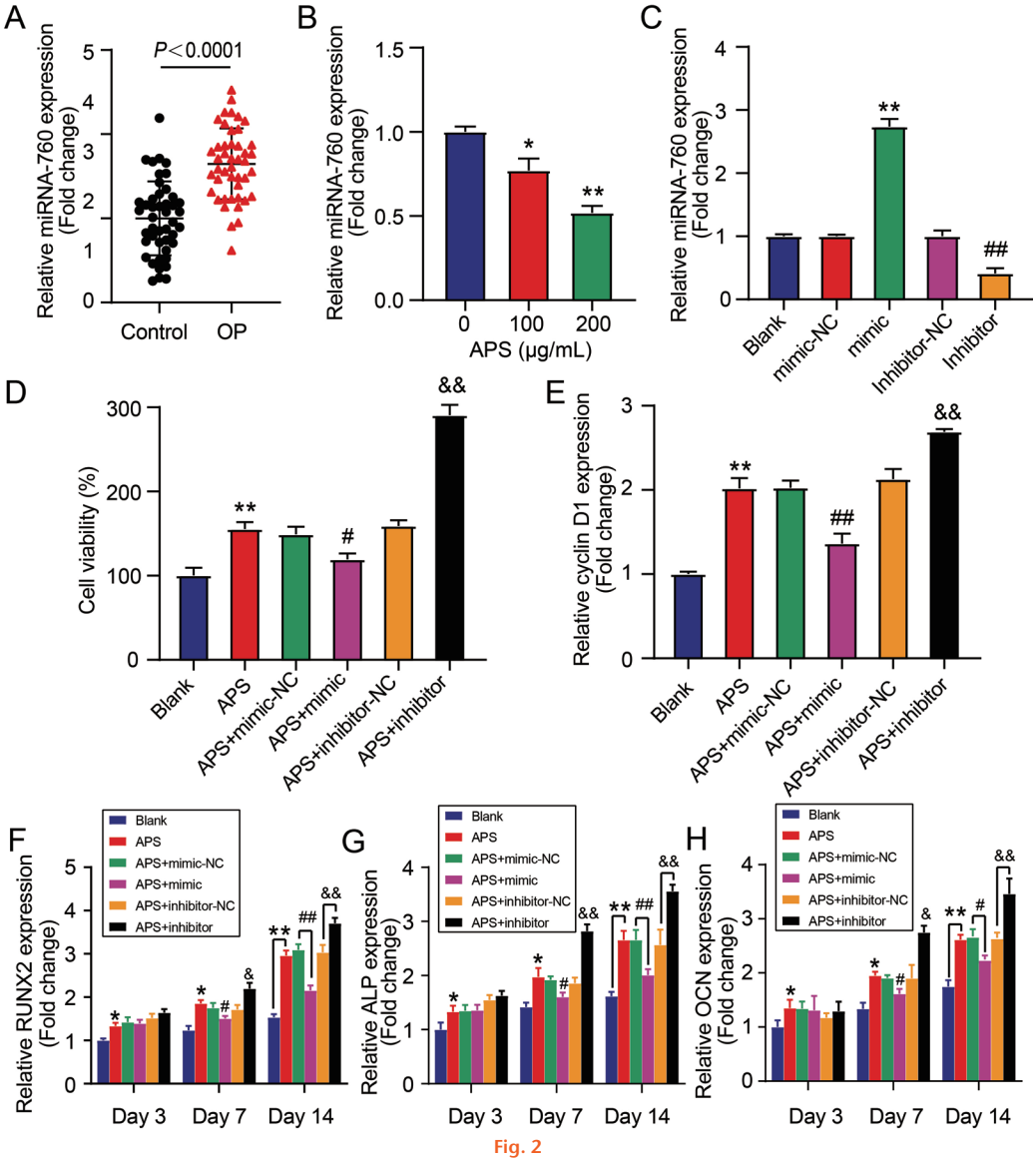

Fig. 2

MicroRNA (miR)-760 was overexpressed in osteoporosis (OP) tissues and diminished the promotive effect of astragalus polysaccharide (APS) on the osteogenic differentiation and proliferation of human bone mesenchymal stem cells (hBMSCs). a) miR-760 levels in bone tissues of OP and fracture patients were determined via quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay. b) miR-760 expression levels in hBMSCs exposed to APS (0, 100, and 200 μg/ml) were assessed via qRT-PCR. *p < 0.05 and **p < 0.001 versus 0 μg/ml. c) miR-760 expression levels in hBMSCs carrying miR-760 mimic or inhibitor were determined via qRT-PCR. **p < 0.001 versus mimic-NC; ##p < 0.001 versus inhibitor-NC. d) Cell Counting Kit-8 assay was conducted to evaluate the viability of hBMSCs transfected with miR-760 inhibitor or mimic combined with 200 μg/ml of APS. **p < 0.001 versus blank; #p < 0.05 versus APS + mimic NC; &&p < 0.001 versus APS + inhibitor NC. e) Cyclin D1 expression levels in hBMSCs transfected with miR-760 inhibitor or mimic combined with 200 μg/ml of APS were analyzed via qRT-PCR. **p < 0.001 versus blank; ##p < 0.001 versus APS + mimic NC; &&p < 0.001 versus APS + inhibitor NC. f) to h) hBMSCs were stimulated with 200 μg/ml of APS after miR-760 mimic or inhibitor transfection. f) Runt-related transcription factor 2 (RUNX2), g) alkaline phosphatase (ALP), and h) osteocalcin (OCN) messenger RNA (mRNA) expression levels were estimated via qRT-PCR at days 3, 7, and 14 of osteogenic differentiation of hBMSCs. *p < 0.05 and **p < 0.001 versus blank; #p < 0.05 and ##p < 0.001 versus APS + mimic NC; &p < 0.05 and && p < 0.001 versus APS + inhibitor NC. All p-values were calculated using one-way analysis of variance, followed by Tukey's Honest Significant Difference post hoc test. NC, negative control.

expression levels of ALP, OCN, and RUNX2, and induced calcium deposition. We found that miR-760 levels were upregulated in OP and downregulated during the osteogenic differentiation of hBMSCs; additionally, miR-760 expression was downregulated by APS. Targeting analyses and further analysis of cell behaviour showed that miR-760 was involved in the osteogenic differentiation of hBMSCs via ANKFY1 targeting.

OP has become a major social problem in countries with an ageing population. Current treatment strategies for OP (e.g. bisphosphates, calcitonin, and oestrogen) still have some clinical side effects.[8,35] MSCs are undifferentiated cells with multilineage differentiation capabilities that have been used as seed cells to develop novel therapies for OP at the cellular level.[36] APS has been shown to have a positive effect on OP remission and

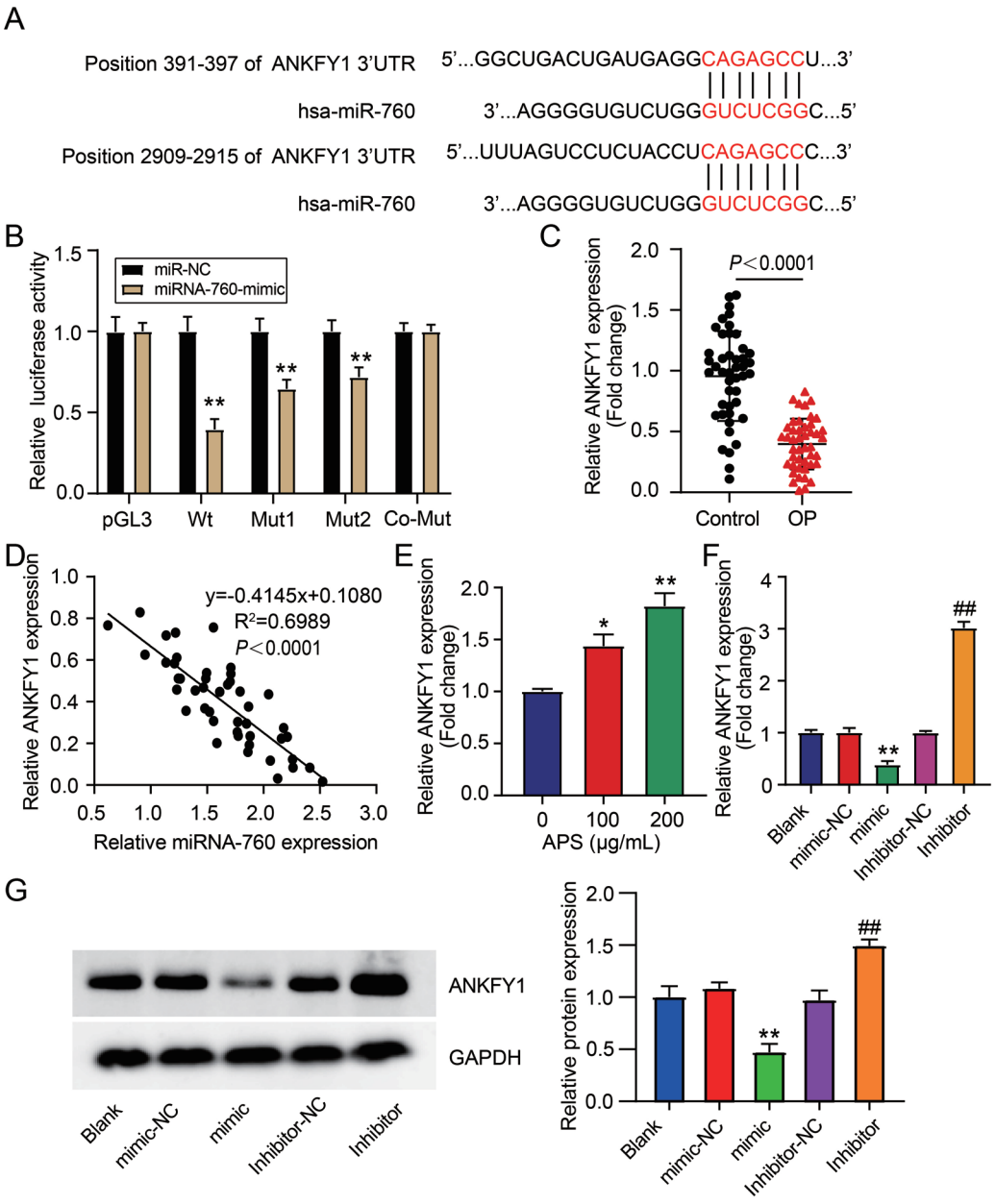

Fig. 3

Ankyrin repeat and FYVE domain containing 1 (ANKFY1) was the target of microRNA (miR)-760. a) TargetScan predicted the relationship between the miR-760 and ANKFY1 sequences. b) Luciferase activity was detected in human bone mesenchymal stem cells (hBMSCs) after subjecting them to a combined transfection of miR-760 mimic and a vector containing the wild-type (WT) or mutant (MUT1, MUT2, and Co-MUT) sequences of ANKFY1. **p < 0.001 versus miR-NC. c) ANKFY1 expression levels in the bone tissues of osteoporosis (OP) and fracture patients were determined via quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay. d) Correlation between miR-760 and ANKFY1 expression levels in the bone tissues of OP was ascertained by Pearson correlation coefficient. e) ANKFY1 expression levels in hBMSCs treated with different concentrations of astragalus polysaccharide (APS) (0, 100, and 200 μg/ml) were estimated via qRT-PCR. *p < 0.05 and **p < 0.001 vs 0 μg/ml. f) The ANKFY1 messenger RNA (mRNA) level in hBMSCs carrying miR-760 mimic or its inhibitor was determined via qRT-PCR. **p < 0.001 versus mimic-NC; ##p < 0.001 versus inhibitor-NC. g) The relative ANKFY1 protein levels were determined in hBMSCs transfected with miR-760 mimic or inhibitor through Western blotting analysis. **p < 0.001 versus mimic-NC; ##p < 0.001 versus inhibitor-NC. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control; UTR, untranslated region. All p-values were calculated using one-way analysis of variance, followed by Tukey's Honest Significant Difference post hoc test.

osteogenic differentiation of MSCs in animal models and cell experiments. OCN and ALP are extracellular matrix proteins generated by osteoblasts that are strongly related to the maintenance of bone homeostasis.[37] Based on these theories and studies, we examined how APS affects the osteogenic differentiation and proliferation of hBMSCs. The osteoblastic differentiation data further demonstrated that APS induced ALP expression and promoted calcium deposition. Combined with the results of Huo and Sun[19] on APS alleviating the effects of

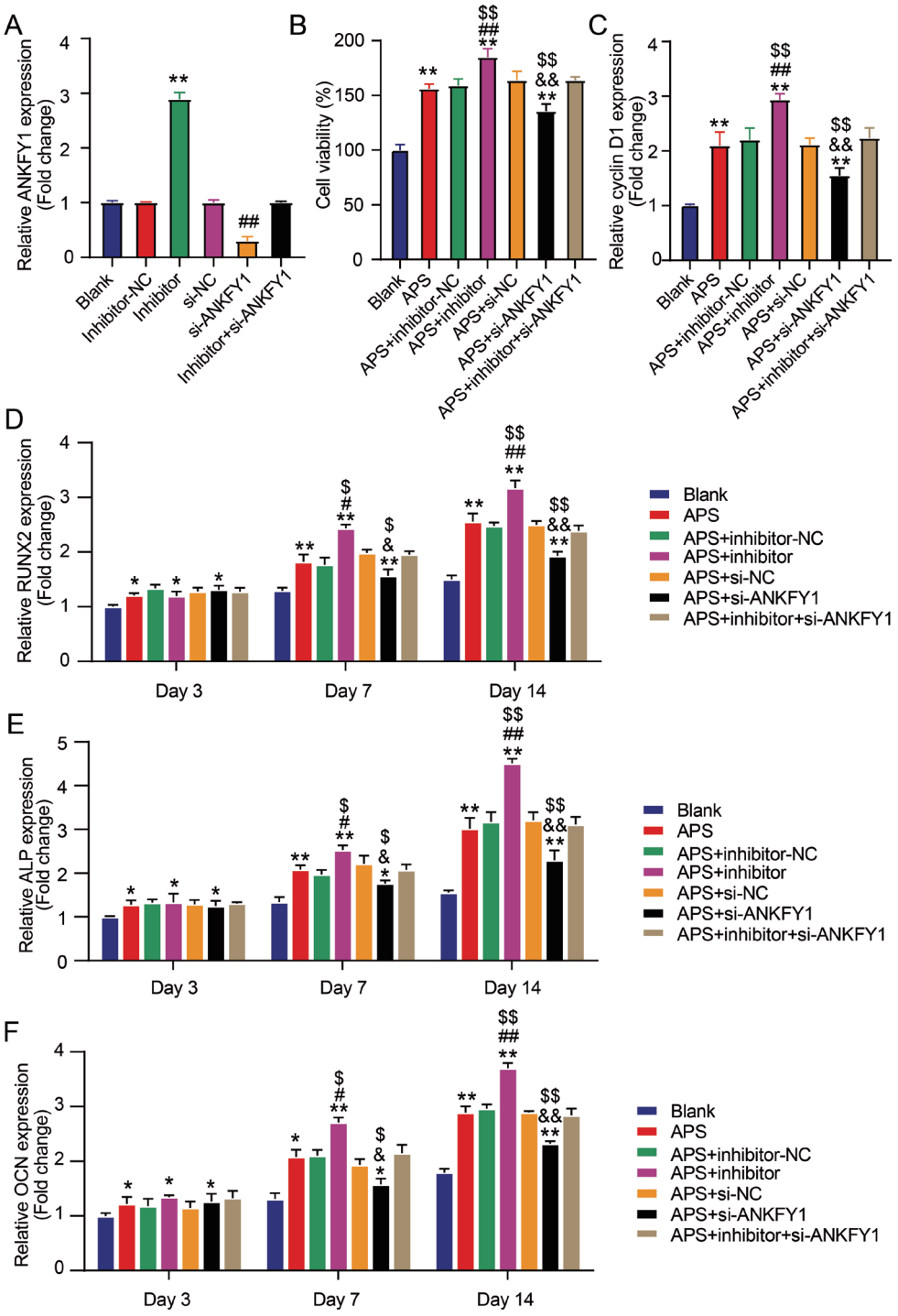

Fig. 4

Astragalus polysaccharide (APS) promotes osteogenic differentiation and proliferation of human bone mesenchymal stem cells (hBMSCs) by boosting ankyrin repeat and FYVE domain containing 1 (ANKFY1) expression via microRNA (miR)-760 inhibition. a) ANKFY1 messenger RNA (mRNA) expression levels in hBMSCs transfected with miR-760 inhibitor and/or si-ANKFY1 were determined via quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). **p < 0.001 versus inhibitor-NC; ##p < 0.001 versus si-NC. b) Cell Counting Kit (CCK)-8 assay was used to assess the viabilities of miR-760-inhibitor- and/or si-ANKFY1-transfected hBMSCs treated with 200 μg/ml APS. **p < 0.001 versus blank; ##p < 0.001 versus APS + inhibitor NC; &&p < 0.001 versus APS + si-ANKFY1; $$p < 0.001 versus APS + inhibitor + si-ANKFY1. c) Cyclin D1 expression levels among the miR-760-inhibitor- and/or si-ANKFY1-transfected hBMSCs stimulated with 200 μg/ml of APS were analyzed via qRT-PCR. **p < 0.001 versus blank; ##p < 0.001 versus APS + inhibitor NC; &&p < 0.001 versus APS + si-ANKFY1; $$p < 0.001 versus APS + inhibitor + si-ANKFY1. d) to f) miR-760-inhibitor- and/or si-ANKFY1-transfected hBMSCs were treated with 200 μg/ml of APS; the mRNA levels of d) runt-related transcription factor 2 (RUNX2), e) alkaline phosphatase (ALP), and f) osteocalcin (OCN) were quantified via qRT-PCR at days 3, 7, and 14 of osteogenic differentiation of hBMSCs. *p < 0.05 and **p < 0.001 versus blank; #p < 0.05 and ##p < 0.001 versus APS + inhibitor NC; &p < 0.05 and &&p < 0.001 versus APS + si-ANKFY1; $p < 0.05 and $$p < 0.001 versus APS + inhibitor + si-ANKFY1. NC, negative control. All p-values were calculated using one-way analysis of variance, followed by Tukey's Honest Significant Difference post hoc test.

postmenopausal OP, we propose that APS could alleviate the effects of OP by promoting osteogenic differentiation of hBMSCs.

miR-760 has been observed to suppress tumours and function as an anticancer factor in various cancers,[38] and it has also been reported to be a potential biomarker for tumours.[38] Recent studies have uncovered the effects of miR-760 on bone diseases. Tang et al[39] discovered the upregulation of miR-760 expression in tissues from patients with rheumatoid arthritis. miR-760 overexpression inhibits C2C12 myoblast migration, differentiation, cell cycle progression, and proliferation. Moreover, Ren et al[24] demonstrated that miR-760 aggravates OP progression and induces the proliferation and osteogenic differentiation of hBMSCs. Consistently, this study also reported low expression levels of miR-760 among patients with OP. We revealed the effects of APS on hBMSC behaviour by modulating miR-760 expression levels. Interestingly, the results showed that APS reduced miR-760 expression in a dose-dependent manner. Furthermore, the promotive effect of APS on the osteogenic differentiation and proliferation of hBMSCs was inhibited by miR-760 overexpression but enhanced by miR-760 knockdown. These data suggest that miR-760 participates in APS regulation and plays a crucial role in the osteogenic differentiation and proliferation of hBMSCs.

ANKFY1 is a membrane-binding protein that does not have a typical transmembrane hydrophobic domain.[26] Instead, it has a coil structure and consists of a BTB/POZ domain located at the N-terminus, multiple anchor proteins in the middle, and a FYVE-finger motif located at the C-terminal.[26] Studies have shown that ANKFY1 is widely expressed during development and among adults; it also participates in different types of endocytosis.[40] However, the effects of ANKFY1 on OP and osteogenic differentiation of hBMSCs are yet to be reported. In this study, we proposed for the first time that ANKFY1 expression was downregulated in OP tissues and upregulated by APS administration in a dose-dependent manner, which could promote the osteogenic differentiation and proliferation of hBMSCs in vitro. In addition, based on targeting analysis, we elucidated the regulatory role of miR-760 in ANKFY1 expression. In view of the rescue analyses, we revealed that ANKFY1 release may modulate the effects of APS on hBMSC proliferation and osteogenic differentiation induced by miR-760.

Recently, some studies have revealed that circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) participate in OP progression by regulating osteogenic differentiation of hBMSCs.[41,42] Therefore, the existence of a circRNA or lncRNA upstream of the miR-760/ANKFY1 axis that regulates OP progression is worthy of in-depth exploration. Meanwhile, some signalling pathways have been shown to be associated with the development of OP, such as the phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt)/glycogen synthase kinase 3 (GSK3) and Wnt/β-catenin signalling pathways.[43,44] The downstream mechanism of ANKFY1 remains unclear because of the lack of literature regarding its effect on osteoblast differentiation. Therefore, the downstream signalling pathway of the miR-760/ANKFY1 axis requires further study. In addition, further in vivo research on the APS-mediated regulation of the miR-760/ANKFY1 axis, which affects the occurrence and development of OP, should be the focus of future studies.

In conclusion, our results indicate that APS could accelerate the osteogenic differentiation and proliferation of hBMSCs via the inhibition of miR-760 expression and upregulation of ANKFY1 expression. This study therefore provides a valuable theoretical basis for the treatment of OP.

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